# An investigation of the microbial enrichment of carob bean residue for use as animal fodder.

Cumming, R. H.

*Award date:*
1974

*Awarding institution:*
University of Bath

# An Investigation of the Microbial Enrichment of Carob Bean Residue for Use as Animal Fodder.

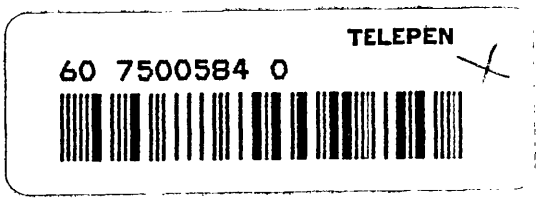
submitted by R.H. Cumming

for the degree of Ph.D

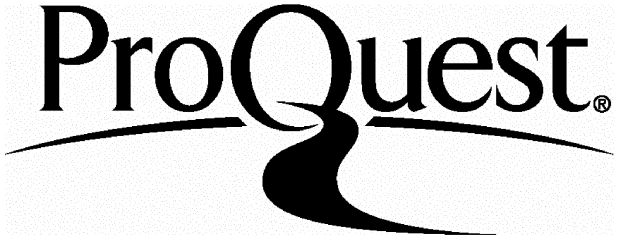
of the Bath University of Technology

1974






....R.H. Cumming.....



I like work : it fascinates me. I can sit and look at it for hours. I love to keep it by me : the idea of getting rid of it nearly breaks my heart.

Jerome Klapka Jerome (1859-1927)

Three men in a boat. Ch. 15.

Summary

The composition of kibbled and spent carob was determined by a gravimetric analysis and confirmed by an alternative colourimetric method. Kibbled carob comprised : 62% ethanol soluble material, 14% water soluble material, 14% lignin, 5.5% cellulose and 6.6% hemicellulose. The composition of spent carob was 11.3% ethanol soluble material, 28% water soluble material, 35% lignin, 14% cellulose and 11% hemicellulose. The suitability of using spent carob in slurry fermentations with Aspergillus niger M1 was examined. A number of nitrogen sources were tested in 2% spent carob slurries. Ammonium sulphate was chosen for further studies and the optimum concentration of this nitrogen source was sought. Evidence is presented for carbohydrate starvation in 2% spent carob slurries when the nitrogen source was not limiting. Protein production was low, with a maximum yield of 10g protein/100g spent carob, the residue containing 15% protein. Attempts to render the spent carob more assimilable to the fungus by ball milling and alkali-swelling were unsuccessful. Trichoderma koningii M223 was used in a solid substrate fermentation of spent carob producing a residue containing only 8% protein. Trichoderma koningii was shown to produce a cellulase.

When Aspergillus niger M1 was used to ferment slurry fermentations of kibbled carob, very high yields were obtained.

Eleven grams protein/100g substrate were produced, and the residue contained 27% protein. Combinations of carob concentrations from 2 to 18% and ammonium sulphate concentrations of 0.35% - 2% were examined for their effect on the yield of protein.

The tannic content of spent and kibbled carob was found to be 2.2% and 1.3% respectively. Autoclaving the carob was shown to increase the apparent tannin content.

The future of the project is discussed.

CONTENTS

MATERIALS AND METHODS Cont'd

RESULTS

# INTRODUCTION

## A. THE CONCEPT OF SINGLE CELL PROTEIN

Single cell protein (SCP) is a euphemism for microbial protein. An association of food and microbes is considered detrimental to the promotion of SCP for human consumption, hence the term "SCP" was coined (Scrimshaw, 1968). This is one particular problem which does not arise when SCP is used for animal consumption. Most reviews on SCP will commence by stating the grave shortage of protein in the world at present or predicted for the future generations of mankind, and how SCP could be the nutritionists' panacea (Litchfield, 1968; Lipinsky and Litchfield, 1070; Snyder, 1970, and Gray, 1970). With the increasing costs of conventional food and animal feed, SCP is fast becoming a commercial proposition and many processes have successfully completed, or are in the process of completing, pilot plant investigations. There are many encouraging calculations on the potential of SCP. Thayson (1956) for instance, has calculated that 1000 lbs of yeast could produce 50 tons of new protein in one day, whereas 1000 lbs of beef animal could only manage to produce 1 lb of new protein per day, and 1000 lbs of soya beans could yield 80 lbs of protein per day (if the total yield is divided by the growing season). Similarly, Gray (1962) has estimated that by growing fungi on the starch of corn grains it is possible to

produce nearly nine times the amount of protein produced by feeding the corn directly to cattle. Some of the advantages of SCP over conventional protein sources can be summarised:

(1) Fast growth rate

(2) Continual production (no seasonal effects)

(3) Utilisation of waste industrial materials as carbon sources

(4) Very little land taken up per production unit.

However, another point of view, as aired by Gaden (1964), is that SCP production usually necessitates a fairly high degree of technology: stirred tank fermentors with controlled aeration rates, pH metering and some form of cell harvesting, of which the simplest is filtration (used with fungi) but could be a more technical process (e.g. centrifugation, flotations, as needed for bacteria and yeasts). Such sophistication would not suit underdeveloped countries where skilled labour is at a premium, unless a team of highly skilled operators could produce SCP on a sufficiently large scale to feed many. The Cassava Process envisaged by the Tropical Products Institute (T.P.I.) circumvents this drawback by deliberately keeping the

fermentation of cassava as simple as possible in a form already familiar to many underdeveloped countries - solid substrate fermentation (Stanton and Wallbridge, 1969).

The choice of micro-organism(s) for the production of SCP is naturally dictated to a large extent by the available carbohydrate source (e.g. algae for $CO_2$ utilisation). There must be a soluble nitrogen source in the substrate for the micro-organisms to convert into cell protein. This nitrogen may be already present in the substrate, or supplied by the addition of inorganic nitrogen salts. The types of substrates used by micro-organisms is very wide indeed, but obviously substrates which have no other commercial use are of prime interest. Industrial wastes often fall into this category, with the added advantage that any SCP production process can often reduce the Biological Oxygen Demand (BOD) of the industrial effluent as well as producing protein. The three main groups of carbon sources used for SCP production are carbohydrates, hydrocarbons and carbon dioxide. Algae have been used for SCP production with carbon dioxide as the major carbon source (Snyder, 1970). These organisms are photoautotrophs and require only carbon

dioxide as a carbon source, together with inorganic salts (one of which supplies the nitrogen), water and light. This literature survey will be confined to the utilisation of carbohydrates for SCP production by fungi. Recent reviews on the production of SCP from hydrocarbons, include Klug and Markovetz (1971) and Humphrey (1970). Fungi are the most widely used group of organisms for SCP production from carbohydrates. (Reviews: Thatcher, 1954; Gray, 1966(a) and (b); Gray, 1970; Snyder, 1970; Battacharjee, 1970 and Kihlberg, 1972). In addition to their ability to use a wide range of substrates, they are much more easily harvested from culture broths than either bacteria or algae (Freeman, 1964).

## E. FUNGI AND FOOD

### (i) The nutritional value of fungi

Fungi can be used to improve the nutritional value of foods or feeds by (1) supplementing a conventional food or feed with mycelium grown in a separate process, (2) fermentation of a substrate and feeding the whole residue to animals or humans, (3) feeding mycelium alone as a complete food. The ability of a fungus to act as the sole component of a food or feed appears to be in dispute. Foster (1949, a) made the rash claim that fungi are unable to supply all the nutritional

requirements of an animal. This claim was based on the trials of workers such as Takata (1929) who found that although mycelium of Aspergillus oryzeae contained 38% protein and was rich in B vitamins, it was not suitable as a complete animal feed. More recently, Church et al (1972) found it necessary to supplement the mycelium of Trichoderma viride with methionine for the sustenance of rats fed the mycelium over a three week period. However, fungi have been fed exclusively to mice with no ill effects (Vinson et al, 1945; Gray, 1962). Most feeding trials have used fungal mycelium as a supplement to conventional feeds, complementing existing deficiencies in the material. Gorcica (1935) found that Aspergillus sydowi was deficient as a source of protein for chicks but proved a useful supplement to whole wheat as an effective source of thiamine and riboflavin. The Protein Advisory Group (1973) claim that fungi have many advantages over other organisms for SCP production e.g. ease of harvesting and more importantly, a balanced amino acid profile with essential amino acids often lacking in other protein sources.

It thus appears that the ability of a fungus to provide all the nutritional needs of an animal is very much dependent on the genus and even the species of the fungus (and perhaps of the animal). The environment in which it is grown can

also have a profound effect. Nielsen and his co-workers (Nielsen et al, 1949) for instance, found that Rhodotorula gracilis mycelium had a similar amino acid profile when grown under different C/N ratios, but the quantities of each amino acid differed. Also, it has long been established (Foster, 1949, b) that the protein and fat content of fungi is largely dependent on the C/N ratio of the medium. Generally, the literature seems to report that many fungi have a well balanced amino acid profile, including the valuable sulphur containing amino acids (Review: Litchfield, 1968). The biological value of microbial proteins is similar to that of plants (Anderson and Jackson, 1958). The vitamin synthesising capacity of fungi is high; yeast being one of the richest sources of B vitamins (Bressani, 1968). Since the cellwalls of fungi are rigid structures, a question arises over the availability of the cell contents to an animal. In some cases it seems necessary to break the cells for maximum utilisation. Tannenbaum and Miller (1967) found that there was an increase in digestibility and net protein utilisation in animals fed broken Bacillus megaterium cells compared with those fed whole cells. It was also found beneficial to disintegrate Spirulina cells before protein extraction and precipitation for a source of SCP

(Hedenskog and Van Hofsten, 1970).

Apart from the use of fungi to provide SCP there have been numerous other uses of fungi in the food industry. Much use has been made of fungi to produce food additives e.g. amino acids (Review: Demain, 1971). Fungi have been used to produce useful metabolites e.g. citric acid (Prescott and Dunn, 1949). An interesting use, originating in the orient, is the production of fermented "cheeses" from soya beans and other crops (Heseltine, 1965). By fermenting these foods with fungi, a characteristic flavour and often a vitamin enrichment is produced (Roelafsen and Thalens, 1964; Rao et al, 1972; see section on solid substrate fermentations).

(ii) Toxicity

Any new source of SCP must be tested for toxicity. One of the disadvantages of SCP is that microbial cells are generally rich in nucleic acids. SCP can contain between 8 and 25 g nucleic acid/100 g protein; for comparison, liver contains 4 g nucleic acid/100 g protein and wheat flour 1.4 g nucleic acid/100 g. Not only does this interfere with digestibility studies necessitating a correction, but also produces toxicity problems per se. For an extensive discussion of this problem see Kilberg's review (1972). Catabolism of nucleic acids results in the production of uric

acid which, in man, accumulates and produces toxic effects. It has been estimated that a safe intake of nucleic acid for a healthy adult should not exceed 2 g/day (equivalent to 10-15 g yeast/day). This will limit the usefulness of SCP for human foods. SCP protein incorporated into animal feeds does not produce the same problems. Many mammals (other than man) are able to convert uric acid to allantoin, a compound which is excreted by the animals thus avoiding uric acid accumulation and its subsequent toxic effects.

Some fungi, of course, produce powerful toxins when grown on certain media (Ciegler and Lilihoj, 1968). These fungi, together with known pathogenic stains should be avoided for SCP production.

## C. REVIEW OF THE LITERATURE PERTAINING TO THE CULTIVATION OF FUNGI AS SCP ON CARBOHYDRATE SUBSTRATES.

It is possible to divide the types of substrates utilised in the production of SCP into three main categories:

(1) Substrates which are totally soluble (e.g. molasses) and are therefore employed in "liquid fermentations".

(2) Substrates which are only partially soluble and thus produce "slurries" for

fermentation (e.g. gelatinised cassava, carob).

(3) Substrates which are largely insoluble and fermented as a pulp (e.g. bran). An insoluble substrate can also be fermented as a slurry.

Substrates of category (2) can also be fermented as a pulp (3) by the judicious addition of liquid. Most of the published work concerns the production of fungal protein from soluble or largely soluble substrates i.e. "liquid fermentations". The efficiency (or sometimes conversion efficiency) of a fermentation is the yield of mycelium produced per 100 g of sugar used, but if the yield is expressed per gram of sugar, then it is the economic co-efficient. Sometimes this sugar is estimated as reducing sugars, other times as total sugars. The efficiency can also be expressed on the initial sugar (reducing or otherwise) present in the medium. The protein efficiency is the amount of protein produced per 100 g sugar used (reducing or otherwise) and again can be expressed in terms of the sugar present initially. In this survey the method used to calculate protein contents is Kjeldhal nitrogen x 6.25, unless stated otherwise.

(i) Soluble substrates

One of the cheapest methods of SCP production is as a by-product of an existing industry. Thus food yeast has been obtained as a by-product of breweries (Dunn, 1952). The waste mycelium from the penicillin industry has successfully been tried as an animal feed supplement (Doctor and Kerur, 1968) and Penicillium chrysogenum substituted mycelium for soya bean meal in feed for mice without ill effects (Pathak and Seshadri, 1965).

During the second world war there was a great stimulation of research into the production of yeast as an SCP source in its own right and the German Yeast Industry was perhaps most advanced. Whey was one of the first substrates to be used. Cheese whey is an industrial waste containing 5% lactose, 0.9% nitrogenous compounds and small amounts of vitamins. It cannot be fed directly to animals as the high lactose content would upset the digestive system. Using lactose fermenting yeasts, SCP containing 54% protein was produced (Muller, 1949). Later work by Graham and his co-workers (Graham et al, 1953) described a feasible process for SCP production from whey producing a marketable product of food yeast rich in protein and

vitamins; at the same time reducing the BOD of the effluent. In this study a mixed culture of Lactobacillus bulgaris and Candida ulitis was employed. Saccharomyces fragilis was soon to take over SCP production from whey and has formed the basis of a viable industrial production plant (Wasserman et al, 1958 and Powell and Hobe, 1964). Progress from this batch production of SCP to a continuous process using Trichosporon cutaneum produced a greater cell yield and higher conversion efficiencies (Atkin et al, 1967). Cheese whey has also been investigated as a possible substrate for the submerged cultivation of Morchella spp (Litchfield and Overbeck, 1965). Such a fermentation produced a mycelium of 34.5% protein at a protein efficiency of 8.12/14.6 g protein per 100 g sugar supplied/used, respectively. This compares with the Trichosporon studies (Atkin et al, 1967) where a yield of cells of 22% protein content with a protein efficiency of 14.4/16.2 g protein per 100 g sugar supplied/used was obtained. Thus, although the percentage protein of Morchella is higher than that of Trichosporon, the latter is more efficient in utilising the sugar supplied in the medium, as is evident from the smaller difference between protein efficiencies calculated on sugar supplied and sugar used.

Another substrate which has been employed for SCP production is sulphate liquor. This is a waste from wood pulping industries and is estimated to contain 31% carbohydrates of which 80% are hexoses and 20% pentoses. Only 65% of these sugars are fermentable (Eweson, 1936). Candida utilis is the fungus most widely employed as it can utilise both hexoses and pentoses. Food yeast so produced has a high protein content (51-52% protein) and rich in vitamins. Protein efficiencies as high as 20 g protein/ 100 g total sugar used have been obtained (Harris et al, 1948). Once again, the process has the added advantage of lowering the BOD of the effluent. Basidiomycetes have also been cultivated in submerged culture in waste sulphite liquor medium, which was diluted to give 2% reducing sugar and supplemented with 0.5% ammonium sulphate (Reusser et al, 1958,a). Ten basidiomycetes gave yields of 4-19 g protein per 100 g of sugar used (2 - 16 g protein/100 g sugar supplied) with protein contents of 15 - 38%. Similar yields were obtained by Cirillo et al (1960) using four basidiomycetes in a semi-continuous set up, by leaving a portion of mycelium in the fermenter after a run as an inoculum for a subsequent

fermentation. More recently, various sulphite waste liquors have been evaluated for the growth of morel mushroom mycelium (Kosaric et al, 1973). In shake flask experiments, with ammonia- or magnesium-based liquors, yields as high as 83% (on carbohydrate consumed) were attained when the liquor was diluted 1 in 5. This mycelium contained between 28-48% protein and had a satisfactory amino acid spectrum. Thus protein efficiencies of between 24 and 41 g protein/100 g sugar consumed were obtained. Calcium sulphite-based liquors did not support the growth of the fungi. For comparative purposes, a product of 60-70% protein has been obtained in an activated sludge process using Pseudomonas grown on sulphite liquor (Amberg and Cormack, 1957).

Molasses is a by-product of the sugar industry for which there is already demand e.g. production of bakers' yeast (Peppler, 1970). In view of competition for the molasses it is even more important for an SCP process using this substrate to have a high efficiency. Food yeasts of 50% protein are being produced in the U.S.A. on molasses based media (Peppler, 1970). Protein efficiencies of 23 g protein per 100 g reducing

sugar supplied have been obtained (Peppler, 1970). In laboratory shake flask experiments, Reusser and his colleagues (Reusser et al, 1958, a) found that ten selected basidiomycetes cultured in a molasses medium diluted to give 6% total sugars, with 0.5% ammonium sulphate, gave protein efficiencies of 5 - 30 g protein per 100 g glucose used (1 - 21 g protein per 100 g glucose supplied). Tricholoma nudum produced mycelium with the highest protein content (54%), which also produced the mycelium of highest protein content on sulphate liquor (38%) albeit lower than on molasses. Using this fungus Reusser and his co-workers (Reusser et al, 1958, b), studied its growth in relation to carbon sources, nitrogen sources, pH, phosphate concentration and aeration. The mycelium was non-toxic to animals and had a vitamin B content that was high enough to maintain normal growth in mice when used at a level of 5% (by weight) to replace vitamins in a synthetic diet. Rhizopus sp. has been reported to produce mycelium with a protein content of 34% when grown on molasses (Shukla and Dutta, 1967). A protein efficiency of 9.5 g protein/100 g sugar consumed was achieved when

molasses was supplied at 6% and ammonium chloride at 0.15%. These workers produced mycelium rich in methione by surface culture techniques. A patent (Szuecs, 1956) commercially exploits molasses for the cultivation of basidiomycetes. Addition of malt sprout extract to a molasses medium containing ammonium hydrogen phosphate produced mushroom mycelium (Agaricus campestris) with the characteristic flavour (Moustafa, 1960). Unfortunately, he gives no data on protein contents of the mycelium, but his data on economic coefficients are similar to those from the work of Reusser (Reusser et al, 1958, a). (Many of these earlier studies were aimed at producing mycelium with a good mushroom flavour rather than a high protein content).

Vinasse, a product from the distillation of fermented sugar-cane juice has been screened for SCP production using ten basidiomycetes (Falanghe, 1962). By suitably diluting the vinasse liquor and adjusting the ammonium sulphate concentration to 0.4%, Falanghe obtained high protein efficiencies for some strains of Agaricus campestris and Boletus indecisus. With

vinasse diluted to 0.55% reducing sugars and supplemented with 0.4% ammonium sulphate, 204 g of mycelium were produced per 100 g reducing sugar used with a protein content of 29%. This is equivalent to 60 g protein per 100 g of reducing sugar used. These particular conditions would provide a very low C/N ratio which would favour the production of a mycelium with a high protein content but usually accompanied by a low mycelium yield (Foster, 1949, b).

Soya bean whey is an industrial waste produced during the separation of soya bean proteins from soya beans. Whey solids, the material remaining after evaporation of whey liquid have a high nitrogen content (3.4%). However, commercial recovery of whey solids is not a feasible process, creating a waste with a high BOD. Falanghe (Falanghe et al, 1964) reduced the BOD of soya bean whey by culturing several basidiomycetes in a whey medium. Tricholoma nudum and Boletus indecisus gave the best yields of 36 and 21 g protein/100 g total sugars supplied, respectively. The protein efficiencies calculated on a sugar used basis are very similar since almost total utilisation of the sugar occurred with these fungi. On increasing the concentration of whey in the medium by

50%, the protein content of the mycelium was decreased from 55% to 35% in the case of T. nudum and 35% to 27% for Boletus indecisus, although in both cases the mycelial yield increased.

Another industrial waste has been exploited by Block and his co-workers (Block et al, 1953) for SCP production, and that is citrus-press water, the fluid extracted from citrus peel and pulp. Yields of fungus equal to that grown on parallel malt extract experiments were obtained (33g of mycelium per 100 g sugar used). However, these yields could only be obtained if the fruit extract was used fresh - it became toxic on storage. Unfortunately, these workers did not report on the protein content of the mycelium grown in the citrus-press water. Using a value of 32.5% protein for the protein content of the mycelium, a figure he quotes has been achieved with other substrates, protein efficiencies of 10 g protein per 100 g sugar supplied were obtained. Yields higher than this have been achieved using the food yeast Candida utilis (Veldhuis, 1952). By propogating the yeast in vigorously aerated citrus-press media cell yields of 26% to 70% were produced (i.e. 26 g cells/

100 g sugar used to 70 g/100 g sugar used). The highest value was obtained with 0.6% initial sugar and the lowest with 3.8% initial sugar. Ninety four per cent of the sugar was used for all concentrations tried.

Litchfield and Overbeck (1965) have investigated certain wastes from the food canning industry for their suitability as a substrate for the submerged culture of Morchella spp. Calculated on a reducing sugar basis, protein efficiencies were 10 g protein/100 g sugar supplied (17 g protein/100 g sugar consumed) when pumpkin-extractor waste was used as a substrate and 7 g protein/100 g sugar supplied (11 g protein/100 g sugar consumed) for corn-canning waste. The mycelium from all media contained about 34% protein.

A more recent examination of vegetable wastes for their potential as substrates in SCP production is provided by Janardhanan and his colleagues (Janardhanan et al. 1970). The vegetable wastes employed were from turnips, cauliflowers and cabbages. However, the materials were not used whole,

but rather an extract was prepared from the boiled minced wastes and then filtered. Glucose was then added to concentrations of 1 - 6%. Various species of Morchella were grown in submerged culture using a number of different nitrogen sources. Increases in the concentration of glucose up to 5% showed progressive increases in yields of mycelium. The five species of Morchella examined differed in their yields of mycelium in each media; but each species gave approximately the same yield in the different extracts, perhaps suggesting that the only carbohydrate available was in the added glucose. No information is supplied on the total sugar supplied or consumed. The highest protein content of the mycelium was 26%.

In an effort to mitigate the tendency for inhabitants of sugar cane producing areas of the world to show symptoms of protein deficiency (supposedly because of bad land management, the land being taken up solely by sugar cane crops) Gray and Paugh (1967) examined the feasibility of using sugar cane juice as a carbohydrate source for SCP production, using Cladosporium sp on the basis of previous studies (Gray and Abou-El-Seoud, 1966, a; 1966, b and 1966, c).

Gray expresses his results as total crude protein per litre (TCPL) of the fermentation broth, not in terms of protein efficiencies. In a medium of cane juice diluted to provide 2% sugars and with ammonium nitrate and potassium dihydrogen phosphate as additives, a yield of 1.6 g TCPL was obtained at 13% protein. A maximum yield in a complete medium (including trace elements, etc.) was 2.6 g TCPL at 16% protein.

There are numerous instances when wholly synthetic media have been used to culture fungi for SCP production. Some will be cited here. Litchfield, Overbeck and Davidson (1963) investigated the production of morel mycelium that would be competitive in price with mushrooms imported from Europe. They found that supplementation of their synthetic medium (glucose based) with corn steep liquor increased the utilisation of reducing sugars. Yields of 10 g protein/100 g sugars supplied (lactose, maltose and glucose) were obtained, with a protein content of 30%. Szuecs (1954 and 1956) disclosed methods for producing mushroom essence and mushroom mycelium in submerged culture and for enhancing the flavour of mushroom mycelia. (Mushroom was used in the sense of basidiomycete, not only the "mushroom"

Agaricus campestris). Interestingly, he used chalk as an insoluble organic supporting material in the media around which mycelial pellets were formed. Humfield and Sugihara (1949 and 1952) demonstrated that Agaricus campestris could be grown in submerged culture and the nutrient requirements were quite simple. Emphasis was on flavour development and it was found necessary to culture the fungi for 3-4 days after exhaustion of the nutrients had occurred for maximum flavour development. A maximum yield of 10 g of mycelium per litre of medium was obtained when urea was supplied at 0.1%. The crude protein content of the mycelium was 36%, which could be increased to a maximum of 42% by a threefold increase in the nitrogen level. No figures are given for efficiencies, but the final protein concentrations were equivalent to 6-8 g TCPL. However, these results must be reviewed with caution, since the strains of Agaricus campestris used by Humfield and Sugihara, namely NRRL 2334, 2335 and 2336, have since been identified as Beauvaria tenella (Molitoris, 1963). This would explain the difficulty in obtaining a mushroom flavour.

Using a purely synthetic medium with glucose as the carbohydrate source, Gray and his co-workers (1964)

screened 175 fast growing fungi imperfecti for their potential as an SCP source in shake flask experiments over a four day period. Highest yields of protein were obtained with Pestalotia yielding 170 mg protein/flask (8.5 g TCPL) with an economic coefficient of 0.67.

By using a cheap source of glucose the overall cost of SCP production in synthetic media might not greatly exceed the cost of a process based on waste soluble carbohydrates, where the costs of collection of enough new material might have to be taken into account. Reviews on the large scale cultivation of higher fungi have been prepared by Robinson and Davidson (1959) and Worgan (1968).

(ii) Slurry fermentations

The fermentations so far cited have one main attribute in common - there are no appreciable undissolved solids in the medium. This may have been achieved by filtration. Media containing small amounts of undissolved solids behave similarly to all-liquid media in terms of ease of aeration. However, if the medium has a high undissolved solid content, aeration rates, adequate for fungal growth, become difficult to achieve. If the solids are not attacked by fungi or if they are susceptible to enzyme attack but are not completely degraded by the end of the fermentation, then

the harvested material will contain mycelium and solids. The latter will have a diluting effect on the percentage protein of the residue. Most of the work in this field has been done by Gray and his associates.

Gray and Abou-El-Seoud (1966, a) investigated the use of sweet potatoes as a substrate for SCP production. The sweet potato has a high carbohydrate content (28%) but a low protein content (1.8%). The material was minced and supplied at a level to give 2% hexoses in a mineral salts medium containing ammonium nitrate as a nitrogen source. The medium also contained 0.2% corn steep liquor. As previously mentioned, Gray does not express his data in terms of protein efficiencies but as TCPL. In the sweet potato fermentation yields of 3-5 g TCPL were achieved with Cladosporium spp. This was equivalent to 25.6 g protein/ 100 g sweet potato, and the residue contained 39% protein. Thus, the protein content of the sweet potato was increased five times over the value of 6.0% for unfermented residues. Addition of corn steep liquor (0.2%) did not significantly increase the TCPL over a medium without this addition, but increasing the corn steep liquor to 0.4% had the effect of increasing the yield of TCPL by 13.7%.

Using a similar method these workers explored the possibility of using manoic (cassava) roots as a substrate for SCP production (Gray and Abou-El-Seoul, 1966, b). As with sweet potatoes, the carbohydrate content of cassava is high, 32% (wet weight basis), and the protein content comparatively low, 0.7% (on a wet weight basis). In order to obtain a medium with a 2% hexose content (as with sweet potato) the amount of fresh cassava roots needed made the medium too dense for adequate aeration. For this reason a final concentration of 5% was used ( 1.6 g carbohydrate). This problem was not encountered with cassava flour which was supplied at a level of 3% ( 7% hexose). From fermented fresh cassava root experiments Cladosporium sp produced a yield of 1.95 g TCPL with a protein content of 20%, a seven-fold increase in percentage protein, and an increase in total protein of six fold.

These same workers tested minced, whole sugar beets and beet shreds as potential substrates for SCP production (Gray and Abou-El-Seoud, 1966, c). In the beet process for the production of sugar, whole beets are crushed and the juice obtained evaporated to a syrup from which sugar is crystallised. Two by-products are

produced (1) the residual syrup "beet molasses" (equivalent to cane molasses of the cane sugar industry), and (2) beet shreds (pulp) which is the residue of pressed beets (equivalent to bagasse from the sugar cane industry).

Several fungi imperfecti were grown in a medium containing whole beets and a species of Cladosporium was found to produce the highest yields. Some of the data is given in table 1. It can be seen that although culture media with the highest beet concentration produced the highest yields of protein in terms of protein concentration (TCPL), more protein per unit beet was obtained in the slurries containing less initial beet.

| Sugar beet g/L | $NH_4Cl$ g/L | TCPL | Protein/ 100 g beet | % crude protein* |
|---|---|---|---|---|
| 140 | 2.0 | 4.18 | 2.99 | 20.32 |
| 85 | 2.0 | 2.96 | 3.48 | 23.12 |

* Dried whole beets contain 3.4% protein.

Table 1.

Thus in terms of utilisation of the substrate, low concentration slurries would seem more efficient.

Dried beet shreds contained 20% carbohydrate and 5% protein. Slurries of 10% were too thick to support the growth

of Cladosporium. However, slurries containing 5% beet shreds yielded 3.8 g TCPL, producing a residue containing 10% protein. Although the TCPL obtained with beet shreds is of the order of that obtained with 85 g/L whole beet (table 1), the low percentage protein of this residue would probably render SCP production from beet shreds uneconomical.

Another substrate examined was rice (Gray and Karve, 1966). Rice flour contains about 70% carbohydrates and 10% protein. The protein content of rice is insufficient to fulfil the minimum daily requirement from the daily diet of rice taken as a staple food by many natives of India (Chahal et al, 1972). Various fungi were grown in a medium containing mineral salts with 2-3% rice flour and with ammonium nitrate as the nitrogen source (providing both nitrate and ammonium as suitable N sources). Omission of any of the ingredients reduced the yield. Highest yields were obtained with Trichoderma sp and Dactylium dendroides, the former increasing the protein content of rice by a factor of 2.9, the latter by a factor of 2.3. These gave products of 30% and 27% protein respectively, equivalent to 4.6 g TCPL and 3.7 g TCPL. Later work (Chahal et al, 1972), showed that Penicillium crustosum could convert rice flour

into fungal mycelium containing 30-35% protein. The qualitative and quantitative analysis of the amino acid content indicated it to be satisfactory from the nutritional aspect. The maximum yield was 1.6 g TCPL when potassium nitrate was supplied as the nitrogen source. Omission of vitamins and trace elements from the basal medium (containing 1.5% rice flour as the carbohydrate source) increased both the total protein and essential amino acid content (including sulphur containing amino acids) indicating therefore that these were deletarious to the growth of the fungus, which is a most unusual occurrence.

The substrates so far reported by Gray and his co-workers were largely soluble. A further series of experiments by these workers reports the use of a largely insoluble substrate - wood pulp. Wood pulp is produced in large quantities by paper mills and has little food value on its own. In their first report Chahal and Gray (1969) used waste wood pulp from the kraft process as a substrate. This pulp contained very little lignin and hemicellulose, being largely cellulose. Wood pulp was supplied at a concentration of 1.5% in a complete medium of salts, trace elements and vitamins. The first screening experiments had their emphasis on fungi which brought

about a loss in weight of the harvested residue. Twelve fungi which produced the greatest weight losses were then evaluated for protein on media with urea as the nitrogen source. (The majority of fungi tested produced the greatest weight loss when urea was the nitrogen source rather than potassium nitrate or ammonium chloride). These workers found that Myrothecium verrucaria required different nitrogen sources for maximum protein synthesis and maximum cellulolytic activity. They also concluded that the percentage loss in weight of the residue and the apparent growth of the fungus (as determined visually) have no relationship to protein production. Thus an organism which solubilised the wood pulp extensively, Trichoderma viride (52% solubilised) yielded the equivalent of 1.1 TCPL, and had very little visible growth while another fungus, Rhizoctonia sp solubilised the pulp to a lesser extent, causing a weight loss of 40%, yet yielded the equivalent of 1.54 g TCPL. Minimal weight losses (8%) were caused by Sclerotium sp, but much mycelial growth was evident and yet the protein yield was 0.5 g TCPL. It seems, therefore, that a fungus can yield a residue with a high protein content by either producing a high yield of protein in relation to the amount of unused substrate, or produce a low yield of protein but extensively solubilising

the substrate i.e. reducing the diluting effect of unused substrate. The highest percentages of protein in these residues was increased from the original 1.6% to 16-18% protein. Further studies with some Rhizoctonia spp isolated from different natural habitats showed that these isolates differed in respect of yields of TCPL with one another to a great extent (Chahal et al, 1969). Thus an isolate from turnip root yielded the equivalent of 1.5 g TCPL at 18% protein whilst an isolate from groundnut seeds yielded 0.8% TCPL at 5% protein. Alternative substrates to wood pulp proved of less value for SCP production. Although the percentage of protein in sugar cane bagasse was increased four-five fold the yield was only the equivalent of 0.8 g TCPL; the yield from rice straw was 0.7 g TCPL and that from rice husk 0.6 g TCPL. With these substrates urea was supplied at a concentration providing 600 mg nitrogen/litre. Control experiments with the same concentration of urea and with wood pulp as a carbon source, Rhizoctonia sp produced a residue of 23% protein at a concentration of 2.2 g TCPL. Such a yield is of the order of those obtained in Gray's experiments with the more soluble substrates (vide supra.)

Different forms of cellulosic substrates have also been investigated by other workers (Rogers et al, 1972). Using a number of cellulosic substrates (filter paper, solka flock, ground refuse and wood pulp), Rogers and his colleagues investigated the growth of eleven cellulolytic fungi in a medium containing 0.2% ammonium chloride as the nitrogen source. They also examined the effect of pretreating the substrates by procedures designed to render the cellulose more susceptible to enzymic attack viz. alkali swelling, high temperatures, electron irradiation and nitrite-photochemical treatment. When the degradability of untreated and alkali-swollen cellulose was compared, the degradation rates of alkali treated cellulose equalled or surpassed that of the untreated cellulose. Four out of eleven fungi completely solubilized both untreated and alkali treated cellulose in 4 days (note that in these cases there would be no cellulose left at the end of the fermentation to dilute the protein content of the mycelium). With the other fungi partial degradation occurred only with the alkali treated cellulose. When the degradability of untreated and high temperature treated cellulose was compared, the untreated cellulose was more degradable. A similar lack of enzymic attack was observed on the irradiated samples. However,

nitrite-photochemical process met with more success. Times required for solubilisation of the substrate by one fungus in particular, Aspergillus fumigatus, decreased from 4 days to 1 day when the cellulose was treated in this way. This fungus was also found to contain all the essential amino acids. The yield of the mycelium was 0.5-0.8 g/g cellulose and total solubilisation of the cellulose has occurred. Protein estimates (4-13%) were low in comparison to previously cited literature values, but these figures were not arrived at by multiplying Kjeldhal nitrogen by 6.25, but rather by summation of amino acids and thus were free from interference from non-proteinaceous nitrogen (e.g. nucleic acids and chitin) which lead to over-estimation in the Kjeldhal N x 6.25 calculations. For comparative purposes, Rogers and his colleagues quoted the protein content of cereal grains (10-15%) estimated by the same method. One of the fungi tested, Aspergillus fumigatus, had a protein content within this range. Aspergillus niger, a fungus of great importance in the carob studies (wide infra), was unable to utilise any of the cellulosic substrates, although these workers report that the mycelium contained 36% protein when grown on starch waste (no details given). Cellulosic material contains regions with a high degree of crystallanity which are not very susceptible to enzymic attack (Reese, 1963).

Attempts to render these crystalline segments more amenable to cellulase action are either physical or chemical procedures. Ball milling or a more thorough grinding procedure (vibrator mill) has met with some success (Katz and Reese, 1968). If cellulosic material containing lignin is finely ground, a further beneficial effect is obtained in that the solubility of the lignin is increased (Pew and Weyna, 1962). The ligno-cellulose complex, which is thought to impede physically the access of cellulase, is also dissociated to some extent by milling procedures (Pew and Weyna, 1962). Partial solubilisation of the lignin and a swelling of the cellulose (making it more permeable to cellulase enzymes) can be achieved by soaking the cellulosic material in sodium hydroxide (Pew and Weyna, 1962; Takrow and Feist, 1969; Pidgen and Heaney, 1969; Doneffer et al, 1969 and Rogers et al, 1972.)

A combination of physical and chemical processes has been employed by a group of workers from Louisiana State University who subjected sugar cane bagasse to mild alkali and heat treatments for SCP production using the bacterium Cellulomonas (Dunlap and Callihan, 1969; Srinivasan and Han, 1969; Dunlap et al, 1970; Callihan, 1970 and Han et al, 1971). These treatments increased the

susceptibility of a variety of cellulose substrates to Cellulomonas degradation and later to a mixed culture of Cellulomonas and Alcagines (which had a synergistic effect on the rate of conversion of cellulose into SCP by Cellulomonas). A fodder of 52% protein was produced at a competitive price.

Updegraff (1970) used ball-milled newspaper as the carbon source for Myrothecium verrucaria. Firstly, in a preliminary screening experiment, none of the bacteria isolated from the soil could synthesise protein at a faster rate than three of the soil fungi isolated, M. verrucaria, Aspergillus fumigatus and Trichoderma lignorum. Studies in aerated stirred jar fermenters produced a maximum rate of protein biosynthesis of 0.3 g/L/day and the maximum yield was 1.42 g/L using 4% ball milled newspaper as a substrate. These estimates of protein were by the Biuret method which suffers less from non-protein nitrogen interference, than does the Kjeldhal N x 6.25 method. From one litre of medium with diammonium phosphate as the nitrogen source and containing 4% ball milled newspaper, a residue of 33.7 g of material was obtained containing 3.3 g protein (organic N x 6.25) or 1.42 g protein if the

Biuret method was used. These percentage protein values (5-10%) are much lower than many already mentioned, but on the other hand a high TCPL was produced.

Two brief reports by Reade and his co-workers (Reade et al, 1972 and Smith and Reade, 1973) revealed the use of fungal upgrading of barley grains. The medium contained 5% barley, 0.1% urea and 0.2% potassium dihydrogen phosphate. The protein content of the fermented residues varied from 8% with Geotrichum candida to 23% with Aspergillus oryzeae. Substitution of ammonium sulphate for urea in a 1% barley medium increased the yield from 3.5 g TCPL to 5.0 g TCPL. However, only 50% of the available nitrogen was used. One hundred per cent utilisation of N was achieved by doubling the barley content of the medium. This also brought about a higher (8.5 g TCPL) yield of protein. A further increase (to 10.5 g TCPL) was obtained in a medium containing 3% barley and 0.42% ammonium sulphate. The protein content of this ma terial was 47% and was produced by the fungus utilising 20% of the carbohydrate and 80% of the available nitrogen. Scaled-up experiments in 1000 L fermenters produced material containing 31% protein rich in essential amino acids ( 2.9 g TCPL). The material was readily eaten by pigs.

An alternative approach for utilising a partially insoluble/totally insoluble substrate for SCP production, rather than fermenting it in a slurry or as a pulp, is to hydrolyse it and ferment the solubles obtained. El-Nawaway (1970) hydrolysed bagasse pith with dilute sulphuric acid and neutralised the hydrolysate with calcium carbonate. Saccharomyces ceriviseae did not grow as well as Candida utilis which could utilise the pentoses as well as hexoses. From 1 kg of pith 172 g of C. utilis cells containing 48.5% protein were obtained ( 84 g protein/kg pith). El-Nawaway suggested dilution of the yeast with residual pith fraction by 70% to produce a valuable fodder with little wastage of bagasse pith. Comparing these figures with those of the Louisiana University group using Cellulomonas to ferment bagasse, where 10 lb bagasse produced 4 lb of cells at 50% protein (i.e. 2 units protein/ 10 units bagasse), El-Nawaway's process is less efficient as it produced 0.8 units protein/10 units bagasse. However, it might be more expensive to subject the bagasse to alkali swelling and heat treatment than El-Nawaway's acid hydrolysis and subsequent neutralisation. Glucose has been released enzymatically from solka flock using enzymes prepared from Trichoderma viride (Ghose and

Kostek, 1970 and Ghose, 1969). However, such a process has yet to be used for the production of substrate for SCP production.

Jarl (1971) shows how a two stage production has been applied to the utilisation of potato wastes in the "Symba Yeast" process. By using a mixed culture of two yeasts an excellent yield of protein has been obtained (43% protein). The process depends on the utilisation of starch present in the potato wastes (effluent from potato processing plants) which contain 10-20% of the potato and therefore have a particularly high BOD. The first yeast Endomycopsis fibulinger, has amylases which hydrolyse the potato starch to glucose, some of which it then consumes for its own growth. The residual glucose is converted to cell protein by Candida utilis which cannot grow on starch itself. The yield of cells from such a mixed culture is greater than that obtained by E. fibulinger alone. Jarl and his colleagues are extending their idea to other substrates. Chrysosporium lignorum breaks down cellulose and lignin with a 60% efficiency (on fibre consumed) but produces a product of only 17% protein. It is hoped therefore to improve the yield by employing C. utilis in a symbiotic process, the yeast using the

glucose produced by the action of Chrysosporium on the cellulosic material.

(iii) Solid substrate fermentations

By definition, fermentations of this category have the substrate in the form of a thick paste, making conventional fermenter techniques for aeration possible. One of the earliest studies on SCP production (Pringsheim and Lichenstein, 1920) used a solid substrate fermentation for the production of fodder during the emergency of the First World War. Ammonium salts were added to straw which was then inoculated with an Aspergillus sp. After a suitable time a cattle feed was produced containing 8% protein - almost nine times the original content of the straw (0.9%).

In this section belongs the most familiar form of SCP production - mushroom cultivation. Mushrooms are generally appreciated for their flavour rather than for their nutritive value, but as Gray (1970) points out, they also compare favourably with vegetables in general with regard to their protein content which is 27% on a dry weight basis. This value (27%) is lower than those obtained by workers cultivating the mushroom in submerged culture. This may be due to the fact that

submerged culture techniques allow more control over both nutrient supply (especially the C/N ratio) and the environmental conditions, essential for optimising yields. Cultivation of the mushroom (Agaricus bisporus) is now a fully established commercial process. The reader is referred to Gray's review (1970) for a comprehensive account. The substrate is a specially composted horse manure which provides both the carbohydrate and the nitrogen sources. After pasteurisation which is achieved by encouraging compost to reach high temperatures ("Peak heating"), the substrate can be "seeded" with mushroom mycelium ("spawn") grown in small scale fermentations. After 15-30 days a thin layer of peat and limestone ("casing") is sprinkled on the surface of the bed, which by now is completely ramified with mycelium. With suitable control of ventilation and temperature, fruiting bodies will develop with 1-8 weeks of the casing process, which is thought to initiate fruiting body production not so much by soil substances released, but by providing an environment antagonistic to the furtherance of vegetative growth, thus stimulating reproduction and the development of fruiting bodies.

The bed will continue to produce successive crops of mushrooms ("breaks") until exhausted - usually after several breaks. Data is not available on the conversion efficiency of such a process, but yields of 1-3 lbs/sq.ft. have been obtained (Gray, 1970). Since horse manure has a valuable fertiliser use as well as a substrate for mushroom cultivation, efforts have been made to cultivate mushrooms on alternative composts.

Block and his co-workers (Block et al, 1965) investigated the use of gumwood sawdust as the principle ingredient for a mushroom compost. He obtained yields of 2-3 lbs/sq. ft., comparable with yields from horse manure composts. On a smaller scale (Block, 1965) other wastes were employed as substrates. These included sewage, newspapers and municipal garbage, which all yielded mushroom in quantity and quality comparable to those of commercial processes.

Using a somewhat similar process to that of the western mushroom grower, the Padi-straw mushroon, Volvaria displasia is cultivated in the orient (Gray, 1970). This process lacks the degree of refinement of its western counterpart and is thus a much simpler

fermentation to manage. Rotting straw which has been soaked in a river for 1-2 days is covered by a layer of dry straw and then inoculated with material from a mature bed. (In more primitive arrangements, no inoculation is used, the process being dependent on chance inoculation from the air flora). Fruiting bodies are produced within 25 days.

In areas of East Asia, Lentinus elodes (the shiitake mushroom) is cultivated on logs of wood which have been soaked in water and had their bark pounded. They are then inoculated by inserting a plug of infected wood into predrilled holes. These inoculated logs are left for 5-8 weeks for the mycelium to ramify the wood and then raised almost to the vertical whereupon fruiting bodies form. As in the Agaricus bisporus cultivation many breaks occur per area of substrate (Gray, 1970).

The orient also possesses many fermented food processes which use fungi. (Hesseltine, 1965, and Stanton and Wallbridge, 1969.) These fermentations often do not produce an overall enrichment with respect to protein (in highly proteinaceous substrates protein may be lost during fermentation) but they often improve digestibility, reduce toxicity and, most significantly, alter the taste (Vankeen and Steinkraus, 1970). The substrates used for these

fermentations are very varied; the more classical fermentations use wheat (tempeh fermentation), maize and soya bean (miso) and soya beans alone (sufu). The changes in the substrate are brought about by a mixed culture of organisms; the air flora sometimes adventitiously serving as an inoculum but more often by passing the substrate through spore laden air. One process which does provide enrichment of the protein content of a substrate is reported by Platt (1964). African locust beans (Parkia filicoidea) after a 3 day fermentation are converted to a production containing 37% protein and rich in riboflavin. However, few experimental details are given.

The Tropical Products Institute have utilised the principles of these oriental fermentations to produce a cheap source of protein. SCP is produced on cassava enriched with a nitrogen source (Brook et al, 1969 and Stanton and Wallbridge, 1969). A controlled environmental (constant temperature and humidity) atmosphere is employed to simulate tropical climates where the process is envisaged being used. The fungi are generally grown in pure culture, work centring round Rhizopus arrhizus and R. oligosporus. Fermentation was stopped when sporulation occurred, yielding a thirtyfold increase in protein from 0.1% to 3%. Parallel experiments in slurry fermentations with 2% cassava produced yields of material with 12-14% protein.

Young (personal communication, 1972) has studied the T.P.I. project from an enzymatic point of view. He examined the production of amylases and glucoamylases by Rhizopus oryeae M180 in both slurry cultures and solid substrate fermentations. The procedure of autoclaving the substrate which gelatinised the starch, had the effect of increasing the protein yield by a factor of three over untreated flour. The cassava was supplemented with urea (1.2%) and mineral salts. After a fermentation period of 3 days 70-80% of the nitrogen was utilised. Dry weight losses were 30% indicating a substantial conversion of substrate to carbon dioxide rather than mycelial substance. Cultures were incubated in a constant humidity and temperature chamber, which also changed the air at set intervals, since adequate aeration was deemed necessary to provide gas exchange. The best yields of protein from the fermented substrate "cheese" was 5.5 g/100 g dry weight cheese at the end of fermentation, 4.2 g/100 g cassava supplied. Attempts were made to grow Candida utilis on the glucose which accumulated during the Rhizopus fermentation. However, protein yields in slurry cultures with both organisms produced lower yields of protein than with Rhizopus alone. Young concluded from his studies that the fungus did not penetrate the substrate to a depth greater than a few millimetres below the surface.

Preliminary trials have been made in the solid substrate fermentation of cellulose for SCP production (Barnes et al, 1972). Using a C/N ratio of 20:1 Sporotrichum thermophile produced a residue which contained 6% protein (dry weight). A moisture content of between 300 and 350% was needed for maximum protein synthesis. (A moisture content of 300% presumably means the substrate contained three times its weight in water, i.e. 75% moisture). These protein contents are low when compared with liquid fermentations, but are very similar to those of the cassava process.

Bagasse has also been fermented as a solid substrate (Cruz et al, 1967). On moistened (60% moisture) residues with ammonium phosphate as a nitrogen source, material containing upwards of 30% protein was obtained with Aspergillus niger and Rhizopus nigricans. This represents an increase in the original protein content of the bagasse by three to fourfold. These yields are excellent even when judged by soluble substrate standards, and are surprising since neither of these fungi are known to produce cellulases.

Solid substrate fermentation : its usefulness and its limitations.

Solid substrate fermentations have not been as freely reported in the literature as liquid fermentations. The over-

riding reason for this is probably the fact that they do not lend themselves to laboratory techniques: maintaining constant environmental conditions for the fermentation is just one handicap (for a discussion of substrate phases see Codner, 1969). On an industrial scale the cassava process (Stanton and Wallbridge, 1969), which is a solid substrate fermentation is envisaged as a simple process requiring little skill for its operation - ideal for underdeveloped countries where technology is limiting. For countries where water is at a premium, solid substrate fermentation, with its lower water to substrate ratio might be a more economical process. However, yields from solid substrate SCP processes have so far been disappointing. Nevertheless, they have been used with great success for fungal metabolite production (Reviews: Hoogerheide, 1954 and Prescott and Dunn, 1959). Enzymes can be produced by solid substrate fermentation. High yielding processes using trays of substrate (Jeffereys, 1948) and rotating drums containing the substrate for better aeration and mixing (Underkoffler et al, 1947) have been used commercially. Hesseltine (1972) has renewed interest in solid substrate fermentations on the laboratory scale, in shake flask experiments for the production of aflatoxin. High yields of both aflatoxin and ochratoxin could be attained by shaking the substrate - not allowing the mycelium to bind the

particles (barley) together. Similar conclusions were reached by Purchase and Nel (1966). Similar techniques for providing substrate aeration might be applicable to solid substrate fermentation for the production of protein.

Another form of solid substrate fermentation is involved in composting. Here the emphasis is on producing a rapid breakdown of plant material in as short a time as possible (Regan and Jeris, 1970). Factors that affect the rate of decomposition might not necessarily produce the highest protein yields, but conditions which show a high weight loss of the substrate will produce less of a diluting effect (due to residual substrate) on fungal protein synthesised. Moisture contents of 50-60% were optimal for the composting process, with 75% for optimal cellulose. As with studies on the utilisation of cellulose substrates for SCP production by the Louisiana group (vide supra), fine grinding of the material produced a faster rate of composting. Although the protein content of solid substrate fermentation may not be greatly increased, the digestibility of the substrate when fed to animals, might be enhanced by fungal action. This was the case when white-rot fungi were grown on straw and sawdust (Hartley and King, 1973).

D. THE CAROB BEAN

The studies reported below are based on the use of

carob pods as a carbohydrate source for SCP production. The carob tree (Ceratonia siliqua) grows in Eastern Mediterranean countries and parts of the USA. It produces fruits, the carob beans (Ceratonia is a legume), which are 6-10 inches long. These beans have a high sugar content (sufficient such that as they dry in the sun some of the sugar drips out of the bean as a syrup). The beans are harvested after this natural drying process and are used for many processes (fig. 1). The carob pod is being evaluated as a substrate for SCP production by Tate & Lyle Ltd. The carob bean comprises the carob pod and the seeds and the components are separated by a kibbling and a sieving process.

(i) Composition of the carob bean

Charalambous (1966) has summarised the data on the composition of the carob pod and seed. This is shown in condensed form in Table 2. Subsequent work by Davies and his co-workers confirms this analysis and also reports the composition of developing beans (Davies et al, 1971). Naturally, the high sugar content of the pod has stimulated commercial interest (cane sugar has about 12% sugars, Gray and Paugh, 1967). More than half of the sugar is sucrose. It can be seen that the carob pod resembles many of the substrates investigated by Gray (vide supra), in that the pod has a high carbohydrate content but a low protein content.

Figure 1.

Some uses of the carob bean

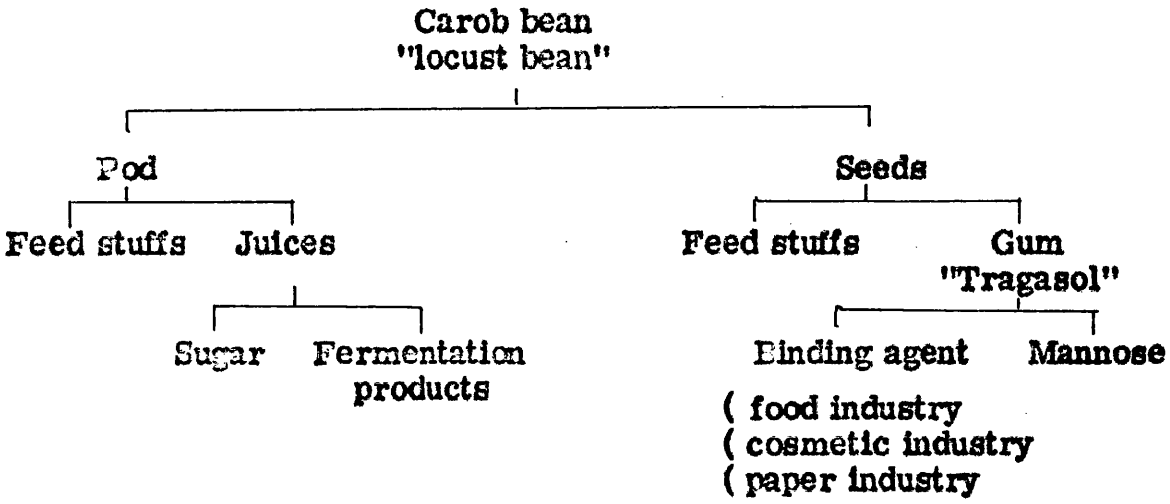



Table 2.

Composition of the carob bean (% dry matter)

| | Pod | Seed |
|---|---|---|
| Crude protein | 5 | 17 |
| Crude fibre | 6 | 9 |
| Fat | 0.5 | 1.6 |
| Total sugars | 55 | 4.2 |
| Reducing sugars | 12 | - |
| Ash | 3 | 4 |

The tannin content of pods has been estimated as 1.4% and the tannins are probably mainly in the condensed form in mature pods (Nachatomi and Alumot, 1963).

(ii) The nutritional value of pods

Carob pods have been reported to depress the growth of chicks (Nachatomi and Alumot, 1963), and the tannins were suggested as the reason and they do have a precipitating effect on proteins (Van Buren and Robinson, 1969), a property which has been used commercially to prepare enzyme concentrates (Patent, 1969). Carob pod extracts lowered the enzymic activity in the rumen (Tagari et al, 1965). Digestibility studies by a number of workers(in Charalambous, 1966) reveal that the degestibility coefficient of carob pods is very low. Maymone and Battaglini (1953) came to the same conclusion when they tested the possibility of using carob bean residues from the alcohol industry as a feed. Bondi and Meyer (1944) claimed that the protein and crude fibre are not digestible by poultry, suggesting that the carob pods can therefore only be considered as a carbohydrate feedstuff. Recently feeding trials (Tate & Lyle, 1970, a) have shown that rats fed on A. niger mycelium grown on molasses increased in weight, while rats fed on A. niger mycelium grown on carob extract lost weight during the same period. It was suggested that extraneous tannin in the carob mycelium might be

the growth depressing factor. The nutritive value of pods can be improved by hot water treatment (Bornstein et al, 1963). Autoclaving and cold water extractions had no beneficial effects, but hot water extraction of the pods seemed to counteract part of the growth-inhibiting effect manifested by stimulation of feed consumption.

The tannins could also inhibit the growth of an organism using carob as a carbohydrate source for SCP production, and indeed, have been shown to inhibit the polygalacturonase activity of Aspergillus niger (Bhutia et al, 1972). Additionally, water extracts of carob deform colony morphology and inhibit growth of micro-organism (Henis et al, 1964) and tannins themselves have been documented from early times to inhibit spore germination (Cook and Taubenhaus, 1911).

## E. THE CAROB PROJECT WITH TATE & LYLE

The utilisation of the carob pod for SCP production is envisaged as a two stage process: (1) a liquid fermentation of carob sugars extracted from the pod and (2) a solid substrate or slurry fermentation of the extracted pods ("spent" carobs). Kibbled carob is extracted with hot water in a two-stage process to remove a large proportion of the sugars. Combination of these extracts with water yields a medium

with 4-10% total initial sugars. Ammonium sulphate and sodium dihydrogen phosphate are added to the liquor. Most of the research has centred around the use of Aspergillus niger (M1) for the fermentation which is usually carried out in stirred tank fermenters, although tower fermenters are also being investigated (Tate & Lyle, 1970, b). Thus the first fermentation process is similar to the fermentation discussed in the section on soluble substrates. Experiments with race plates revealed (1) the optimum temperature for growth was 30-32$^{o}$C, (2) the optimum dilution of the carob extract was one which produced 4-6% total initial sugars, and (3) sea water used for carob extraction did not yield as great as those produced when tapwater was used. (It would be an advantage in arid countries where tap water is at a premium if an SCP process could use sea water as a diluent). (Tate & Lyle, 1969, a). It must always be remembered that these results may not necessarily hold true on a stirred tank fermentation. Large scale fermentation studies (Tate & Lyle, 1969, c) in 40 L fermenters using a carob extract diluted to provide 4% initial total sugars produced a conversion efficiency of 40%, yielding 4.7 g protein per litre after 28 hours. These protein concentrations are similar to many obtained by Gray and other workers (vide supra). During this fermentation the pH of the medium dropped from 4.5 to 1.9. Such a final

pH could be harmful to stainless steel tanks; and, fortunately, fermentations with a controlled pH (by addition of sodium hydroxide) did not produce reduced yields. Interestingly, 4 L fermentation experiments with unsupplemented carob extract adjusted to give 16% initial total sugars produced high conversion efficiencies (40%) and the mycelium was rich in protein (28%) (Tate & Lyle, 1969, d). Expressed in terms of protein efficiency this corresponds to 12 g protein/100 g sugar supplied, a figure only just lower than that obtained with carob media fully supplemented with salts, including 0.38% ammonium sulphate (16 g protein/100 g sugar supplied) (Tate & Lyle, 1969, e). Fermentations using M1 in 50 L stirred tank fermenters revealed an increase in yield of mycelium with increasing initial sugar concentration (i.e. carob extract). Thus by increasing the sugar concentration from 4.7% to 7.7% the yield was increased by 30% although both the conversion efficiency and percentage protein in the mycelium dropped (Tate & Lyle, 1970, c). Contrary results have been obtained in shake-flask experiments (Tate & Lyle, 1969, f), where maximum mycelium and percentage protein were achieved when sugar concentrations of 11-16% were used. The Microbial Research team at the University of Athens (Tate & Lyle, 1969, g) carried out an analysis of the composition of M1 grown under

different conditions. When the total nitrogen content was 4.8% (equivalent to 30% crude protein) nucleic acids estimated as 1.2% N and the cell walls 0.8% N, producing a "protein" nitrogen content of 2.8% N (equivalent to 18% crude protein, a figure nearer the alkali soluble protein estimate of 21% protein). Many other fungi have been screened as a possible source for an SCP carob-based fermentation (Tate & Lyle, 1969, h) with yields comparable to M1. A high yielding Penicillium sp. has been further investigated (Tate & Lyle, 1970, d).

For the maximum utilisation of the carob pod, the spent residues should be used in addition to the extracted carob liquor. The spent carob is not very satisfactory as an animal feed since the only nutritive part (the sugars) have been largely removed. It was thus intended to upgrade the spent carob by a fermentation process which would increase the protein content of the residue and perhaps render it more digestible when used as an animal feed. Preliminary trials have been carried out by Tate & Lyle. A number of organisms were selected by the Rautella-Cowling test on their ability to degrade Walseth cellulose (Rautella and Cowling, 1966 and Tate & Lyle, 1969, i). Solid substrate fermentations were tried using the best of these organisms (Sporotrichum prunosum)

on a medium containing beet pulp and ammonium phosphate moistened to a level of 75%. Visual examination after 7 days showed that the fungus had successfully colonised the substrate. No chemical measurements were made. Further trials (Tate & Lyle, 1969, j) using a number of fungi produced weight losses of 20-47% after 8 days. Weak mycelial growth occurred in a spent carob slurry (30% w/w) when M1 was employed (Tate & Lyle, 1969, k). Very little growth occurred in an orange peel slurry fermentation using M1 (Tate & Lyle, 1972, a), or date waste slurry fermentations (Tate & Lyle, 1972, b). A thick slurry fermentation of spent carob using Polystictus sp and Myrothecium verrucaria proved unsuccessful, little growth of either fungus occurring (Tate & Lyle, 1969, l).

Further experiments concerning the utilisation of spent carob for SCP production and an alternative to the two stage utilisation of kibbled carob are presented here.

# MATERIALS AND METHODS

## 1. PHENOL-SULPHURIC ACID METHOD FOR TOTAL SUGARS

The phenol-sulphuric acid method (Dubois et al, 1965) was used for the determination of total sugars. To a 1 ml sample (0-150 $\mu$g sugars) 1 ml of 5% phenol was added and thoroughly mixed. 2.5 mls of concentrated sulphuric acid were then added, directed at the surface of the mixture in tall-sided tubes, to create as much heating as possible. (A Zipette (Jencons) was found useful for this application since the acid must be added in as short a time as possible). The tubes were then allowed to cool to room temperature and the orange-brown colour produced read in 1 cm glass cells at 490 m$\mu$, using a reagent blank. A standard curve was constructed using known weights of glucose and the region of linearity obtained. Standards within this range were then incorporated routinely in any sugar determination. All samples were diluted if necessary to give an optical density within the region of linearity. Samples were usually in triplicate.

## 2. REDUCING SUGAR DETERMINATION

An adaption of the Nelson-Somogyi method was used. (Somogyi, 1951; Nelson, 1944). Solutions were stored at 30$^{o}$C to avoid crystallisation of the Somogyi reagent. To 1 ml of sample (in triplicate) 3 mls of Somogyi reagent were added and the mixture heated in a boiling water bath for 10 minutes with a glass marble on top of the tube to reduce moisture loss. (Somogyi reagent: 4 volumes A + 1 volume B; A. Rochelle salt 12 g, sodium carbonate (anhy) 24 g, sodium bicarbonate 16 g, sodium sulphate

(anhy) 144 g, water to 800 mls; B. copper sulphate ($5H_2O$) 4 g, sodium sulphate (anhy) 36 g, water to 200 mls.)

The tubes were then cooled under the cold tap and 3 mls of the Nelson reagent added, with vigorous mixing. (Nelson reagent: 25 g ammonium molybdate were dissolved in 450 mls of distilled water on 21 mls concentrated sulphuric acid added with gentle mixing. 3g of sodium arsenite ($7H_2O$) dissolved in 25 mls distilled water were then added and the mixture allowed to stand for 2 days at 37°C before use).

A green colour developed within 15 minutes but a further wait of at least 2 hours was necessary to allow all the gas bubbles to evolve, which would otherwise form on the cuvette sides, making an accurate optical density reading impossible. The evolution of gas could be speeded up if the tubes were incubated at 37°C for 30 minutes with shaking at frequent intervals. The tubes were read at 660mμ against a reagent blank in 1 cm glass cells. A standard curve was prepared using known concentrations of glucose and the region of linearity determined (0-40μg glucose/ml). Samples were diluted to give optical densities within this range. A standard was included with each batch of tubes.

3. NITROGEN DETERMINATION (AS AMMONIA)

Nitrogen was measured as ammonia by the method of Searcy et al (1967). To triplicate samples of 0.5 ml, 1 ml of Reagent A was added, the tubes mixed and then 1 ml of Reagent B added.

(Reagent A: made up to 1 L with distilled water: 85 g sodium salicylate, 0.6 g sodium nitroprusside. Reagent B: made up to 1 L with 0.3N NaOH : 2.5 g sodium dichloroisocyanurate). The green colour was developed for 10 minutes at 30°C. 2.5 mls of distilled water were then added to each tube, which were then mixed and read in 1 cm glass cells at 660 mμ using a reagent blank. A standard curve was prepared from known concentrations of ammonium sulphate, such that the linear region could be ascertained (0-8 μgN ml). Samples were diluted to give optical densities within this range. A standard was included with each batch of tubes.

4. THE MEASUREMENT OF PROTEIN

A. LOWRY METHOD

The Lowry method of protein determination was tried on carob using the adaption of Gorsuch and Norton (Lowry et al, 1951, and Gorsuch and Norton, 1969). To a sample containing not more than 5 mg of protein 10 mls of 1N sodium hydroxide were added and the mixture heated 15 minutes in a boiling water bath using a marble on top of the tubes to reduce evaporation. After cooling to room temperature the tubes were spun at 4,000 rpm on a bench centrifuge. To 0.6 mls of the supernatant 3 mls of the copper reagent were added and the tubes allowed to stand at room temperature for 10 minutes (copper reagent: 1 ml B + 50 ml A freshly prepared; A = 2% sodium carbonate, B = 0.5% copper sulphate ($5H_2O$) in 1% sodium citrate. 0.3 mls of commercial

(BDH) Folin-Ciocalteu's reagent which had been diluted with an equal volume of 1.4 NHCl were added and the tubes rapidly mixed. The optical density of each tube was read in 1 cm glass cells at 760 mμ after incubation at room temperature for 1 hour. A standard curve was prepared using bovine serum albumin.

B. KJELDHAL NITROGEN DETERMINATION

Protein was also estimated by multiplying Kjeldhal nitrogen by 6.25. Kjeldhal nitrogen was estimated at first by the following procedure. Duplicate samples containing not more than 400 μgN were digested with 2 mls of digest reagent (5 selenium-sodium sulphate catalyst tablets (BDH) dissolved in 100 mls warm concentrated sulphuric acid, analytic grade) on a gas-fired micro-digestion apparatus (Gallenkamp). After the reaction mixture had cleared it was heated more strongly for a further 20 minutes to effect the breakdown of more resistant cyclic nitrogenous compounds (Bradstreet, 1965) and allowed to cool. The contents of the digestion flasks were then washed into a beaker which was placed in a small ice bath and the pH brought to between pH 6 and 8, by the addition of concentrated sodium hydroxide, with constant stirring. Neutralisation is necessary for the assay of samples by the Searcy method (Fraser and Russel, 1969). The neutralised digest was made up to 50 mls and 0.5 mls used for the assay of ammonia by the Searcy method (above). Only one Kjeldhal nitrogen estimation was prepared on samples already replicated in growth experiments through experimental design. Standard curves were constructed

from solutions of ammonium sulphate run through the whole procedure.

For the majority of Kjeldhal determinations a modification of the above technique was used. In order to reduce the over-estimation of protein by a total nitrogen technique such as the Kjeldhal method, the contribution to total nitrogen by chitin found in fungal cell walls was eliminated by employing a 1N sodium hydroxide extraction before the Kjeldhal digestion. The sample containing not more than 4 mgN was extracted for 15 minutes in a boiling water bath with 10 mls of 1N NaOH as in the Gorsuch and Norton (1969) technique for Lowry protein determinations. Larger volumes of sodium hydroxide (50 mls) with correspondingly larger samples sizes were employed for the growth experiments with kibbled carob. After this alkali extraction the carob was allowed to settle to the bottom of vessel and 1 ml of the supernatant digested with 1 ml of the acid and the nitrogen assayed in the usual fashion.

5. CELLULOSE DETERMINATION

Cellulose was determined by the method developed by Updegraff (1969). To triplicate centrifuge tubes containing not more than 150 mg cellulose, 3 mls of acetic-nitric reagent were added in 1 ml aliquots with thorough mixing after each addition (acetic-nitric reagent was prepared by mixing 50 mls 80% acetic acid with 15 mls concentrated nitric acid). The tubes, with glass

marbles on top to reduce evaporation, were placed in a boiling water bath for 30 minutes. After cooling the tubes were spun at 3,000 g for 5 minutes and the supernatant discarded. The sediment was resuspended in 10 mls of 50% ethanol and respun. The washing was repeated and the sediment finally suspended in a total volume of 10 mls 67% sulphuric acid, with thorough mixing after the addition of the first few mls. The tubes were then left at room temperature for 1 hour during which the cellulose was solubilised. A sample of this digest, suitably diluted, was assayed for total carbohydrate by the Phenol-sulphuric acid method. A standard curve was constructed from pure cellulose (Whatman cellulose powder), the cellulose contents being estimated as glucose after the hydrolysis in the 67% sulphuric acid.

6. ESTIMATION OF TANNIN

Two methods were used, the first as laid down by the Association of Official Agricultural Chemists (AOAC 1965, a). 5 g of carob was boiled with 400 mls of distilled water for 30 minutes, allowed to cool and then diluted to 500 mls. To a 10 ml sample, 25 mls of indigo carmine solution (Indigo carmine 6 g, concentrated sulphuric acid 50 mls, diluted to 1 L) were added and the mixture diluted with approximately 750 mls distilled water. This was titrated with potassium permanganate solution (1.33 g/L, its equivalent obtained by titration with 0.10 N oxalic acid). The end

point was indicated by a change of blue to a straw colour on the addition of potassium permanganate. (This volume is called A mls). Next, to 100 mls of carob extract 50 mls of gelatin solution were added (25 g gelatin was soaked for 1 hour in saturated sodium chloride, then heated until dissolved, cooled and made up to 1 L). After mixing with saturated sodium chloride, 100 mls acidified sodium chloride was added (975 mls saturated sodium chloride and 25 mls concentrated sulphuric acid). 10 g of powdered kaolin (BDH) was then added and the mixture vigorously shaken. After most of the kaolin had settled the supernatant was spun at 3000 g for 10 minutes to remove the last traces of particulate matter. 25 mls of the supernatant were then added to 25 mls of indigo carmine, diluted with 750 mls distilled water and titrated as before (to give B mls). A-B gave the quantity of potassium permanganate (of whose equivalent with oxalic acid is known) to oxidise the tannin. The amount of tannic acid was calculated from : 1 ml of 0.10 oxalic acid 0.0042 g gallotannic acid.

The second method for tannin estimation included the AOAC method of gelatin adsorption but used the method of Swain and Hillis (1959) to estimate total phenols before and after the gelatin adsorption stage. To a 7 ml sample, made up to 7 mls with distilled water if necessary, 0.5 mls of Folin-Denis reagent were added. (Folin-Denis reagent (AOAC 1965, b),): to 750 mls

distilled water, 100 g of sodium tungstate. ($2H_2O$) 20 g phosphomolybdic acid and 50 mls of phosphoric acid were added and the mixture refluxed for 2 hours. After cooling the volume was made up to 1 L. After 3 minutes at room temperature 1.0 ml of saturated sodium carbonate was added and the tubes were then thoroughly mixed. Incubation for 1 hour at room temperature produced a blue colour which was read in 1 cm glass cells at 725 mμ using a reagent blank. Tannic acid (not exceeding 100 μg per assay tube), was used for the preparation of a standard curve. The tannins in the sample were quantitated by the difference in optical densities before and after gelatin adsorption.

7. THE MEASUREMENT OF CELLULASE ACTIVITY

A method was used which detected the final product, glucose, from the action of the cellulase-enzyme complex on cellulose as recommended by Mandels and Reese (1964). 50 mg of absorbant cotton wool were weighed into tubes and 2 mls of citrate phosphate buffer pH 4.8 were added. 2 mls of enzyme solution were then added, the mixture thoroughly mixed and incubated at 50°C for 1 hour (Mandels et al, 1971). A blank was prepared by incubating 50 mg cotton with 2 mls buffer and 2 mls of boiled enzyme (100°C for 15 minutes). Controls without substrate were incubated with each sample, to determine if glucose could be produced from culture filtrates without cellulose. After the incubation period the tubes were once again

thoroughly mixed and a 1 ml sample assayed for reducing sugar by the method of Nelson-Somogyi (above). pH profiles: culture filtrates were tested for cellulase activity in citrate-phosphate buffers of pH3-8. Time plot: the relationship between glucose production and time was determined by sampling mixtures of enzyme and substrate (pH 4.8) at 30 minute intervals from 0-4 hours. Duplicate tubes were used for each time interval, since sampling from one reaction mixture would alter the enzyme substrate ratio for subsequent sampling owing to the substrate being in solid form. Standard curve: a very active filtrate was diluted by various degrees to determine if a linear relationship existed between enzyme concentration and glucose production.

8. THE MEASUREMENT OF TANNASE ACTIVITY

The method of Iibuchi and his workers was used (Iibuchi et al, 1967). Purified tannic acid was first prepared by the following procedure. 100 mls of a 10% tannic acid solution were extracted four times with 100 mls of ethyl ether and the ethereal layer discarded in each case. The pH of the extracted tannic acid was brought to pH 6.8 with the addition of 2N sodium hydroxide and then extracted four times with 100 mls ethyl acetate. The ethyl acetate fractions were then combined and reduced to dryness on a rotary evaporator at room temperature, the residue resuspended in ethyl ether and air-dried to give a white fluffy powder. 0.2 mls of 0.35%

purified tannic acid in 0.05 M citrate-phosphate buffer pH 5.5 was added to 0.05 mls of enzyme and incubated at 50°C for 30 minutes. The mixture was incubated at 50°C since Aspergillus niger tannase is quite stable at the temperature (Yamada et al, 1968). 0.05 mls of the reaction mixture was then added to 5 mls of 95% ethanol which stopped the reaction. The tubes, in triplicate, were read against a boiled enzyme control (15 minutes at 100°C) at 310 mμ, setting the optical density of the blank to 0.8; enzyme activity producing a reduction in optical density. A modification of this method using 0.5 mls enzyme and 0.2 mls substrate (0.35% purified tannic acid in 0.05 M citrate-phosphate buffer pH 5.5) and adding 5 mls of 95% ethanol to stop the reaction, was found more successful. pH profiles, time plots and concentration curves were prepared in a similar manner to those of the cellulase assay.

9. PHENOL OXIDASE ACTIVITY

The procedure of Kirk and Kelman (1965) was adhered to. 1 ml of enzyme solution (culture filtrate) was added to 1 ml of 40 μM/ml catechol and 3.0 mls of 0.1 M pH 5.5 phosphate buffer. After 1 hour incubation at 30°C the tubes were read at 395 mμ in 1 cm glass cells against a boiled enzyme (15 minutes 100°C) blank with which the spectrophotometer was zeroed. An increase in optical density

was indicative of enzyme activity.

10. GRAVIMETRIC ANALYSIS OF CAROB

The method chosen for a gross analysis of the available carbohydrate substrates in the carob was that used by Jermyn and Isherwood (1956). Each stage in the procedure was repeated at least three times and average valuescalculated.

Preparation of material: Unless otherwise stated, carob was freeze dried and ground to pass a 10 mesh sieve to give material of not greater than 1.7 mm particle size.

Ethanol extraction: 30 g of carob were refluxed with 200 mls ethanol for 1 hour, filtered on a sintered glass filter and the extraction continued with a further 200 mls of ethanol. The process was repeated until the filtrate was sugar free, as determined by the phenol-sulphuric acid method. The last traces of sugar were removed by washing the carob on the filter with boiling ethanol. The combined filtrates and washings were assayed for total sugars by the phenol-sulphuric acid method. The ethanol extracted carob was washed with acetone and dried to a constant weight in a current of air.

Water extraction: 10 g of ethanol extracted material were refluxed with 750 mls of distilled water for 12 hours, filtered and washed with boiling water until sugar free. The combined filtrate and washings were assayed for total sugars by the phenol-

sulphuric acid method and the carob washed with acetone and dried to a constant weight in a current of air.

Holocellulose preparation: 5g of material from the preceding step were suspended in 160 mls of water in a 250 ml Erlenmeyer flask, 10 drops of glacial acetic acid added followed by 1.5 g of sodium chlorite and the flask immersed in a water bath at 75°C. This process of delignification was carried out for 5½ hours in a fume cupboard and with a glass marble on top of the flask to reduce evaporation losses. The carob which was now white in colour, was harvested by filtration on a sintered glass funnel and washed with 500 mls of iced distilled water followed by acetone and finally dried to a constant weight in a current of air.

Hemicellulose A extraction: 2.5 g of holocellulose were placed in a 125 ml Erlenmeyer flask and 100 mls of 1N potassium hydroxide added. The flask was closed with a rubber bung and immersed in a water bath at 20°C for 2 hours, gently swirling the contents at 10 minute intervals. The mixture was then separated on a sintered glass funnel, washed with 50 mls 1N potassium hydroxide and then with 150 mls of distilled water. The filtrates were run into 10 mls of glacial acetic acid. The combined filtrates were then transferred to a 2L flask and the suction flask washed with 25 mls of distilled water (2 x 12.5 mls).

The combined filtrates and washings were then made up to 2L with ethanol and allowed to stand overnight in a cold room ($4^{o}C$). The white flocculent precipitate which formed was harvested by first decanting the supernatant and then by centrifugation of the slurry. The material was washed onto a sintered glass funnel with ethanol, acetone and then ether, to be finally dried to a constant weight in a current of air. The material so collected was called hemicellulose. A.

Hemicellulose B extraction: The residue after extraction of hemicellulose A was returned to the 125 ml Erlenmeyer flask and 100 mls of 4N potassium hydroxide added. Again the flask was stoppered and kept at $20^{o}C$ for 2 hours, swirling at 10 minute intervals. After this treatment the liquor was filtered into 45 mls of glacial acetic acid and the suction flask washed with 25 mls of 4N potassium hydroxide (2 x 12.5 mls). The residue was then washed with 25 mls of 2N acetic acid. The filtrates were then combined and ethanol added as for hemicellulose A. The material obtained from this extraction was called hemicellulose B.

$\alpha$ cellulose: This was the residue after extraction of hemicellulose B and was washed with acetone and air dried to a constant weight.

## 11. CULTURE MAINTENANCE

Cultures of "cellulolytic" fungi were supplied by Tate & Lyle Ltd. and were grown on the medium used for the Rautella-Cowling test for "cellulase" activity (Rautella and Cowling, 1966).

This test involved the use of Walseth cellulose, prepared as follows:

Preparation of Walseth cellulose: Whatman cellulose powder was swollen in 85% O-phosphoric acid for 2 hours at 4°C with regular stirring. The viscous solution was then washed into 4 L of distilled water, whereupon a precipitate formed which, after settling, was collected by centrifugation. The precipitated cellulose was resuspended in 1% sodium carbonate, respun and washed several times with distilled water, until neutral. The cellulose was washed with acetone onto a sintered glass funnel, washed with ether and air dried.

The Rautella-Cowling medium was made up as follows:

| | |
|---|---|
| Potassium dihydrogen phosphate | 0.6 g |
| Ammonium dihydrogen phosphate | 2.0 g |
| di-Potassium hydrogen phosphate | 0.4 g |
| Magnesium sulphate ($7H_2O$) | 0.89 g |
| Difco yeast extract | 0.5 g |
| Thiamine HCl | 100 mg |
| Adenine | 4 mg |
| Agar | 17 g |
| Walseth cellulose | 5 g |

The cellulose was dissolved in 100 mls of distilled water and the other ingredients added in the order given, finally making the medium up to 1L with distilled water. The medium was autoclaved for 15 minutes at 15 lbs/sq. in.

On receipt of the cultures they were subcultured onto fresh Rautella-Cowling agar and incubated at their optimum temperature. For prolonged storage soil cultures were prepared. 2 mls of autoclaved 0.05% Tween 80 were added aseptically to each of the original cultures and the spores and/or mycellium prised off the agar into suspension. This suspension was transferred to soil cultures which were then stored at room temperature.

Preparation of soil cultures : (Greene and Fred, 1934). Soil cultures were made by sieving moist loam through a 3 mm mesh and distributing it in 10 g amounts into universal containers into which 4 mls of distilled water had been added. Sterilisation was achieved by autoclaving for 15 minutes at 15 lbs/sq. in on the three successive days. Confirmation of sterility was sought by plating loopsfull of soil from 10% of the cultures onto malt extract agar (Oxoid) and nutrient agar (Oxoid) and incubating at 27°C for 5 days.

12. SCREENING EXPERIMENTS

Cellulase production: To test their ability to grow on carob the fungi were grown on 2% ball milled (18 hr) spent carob agar containing 0.35% ammonium sulphate and 0.1% sodium dihydrogen phosphate. The extent of their growth was recorded visually. Fungi which grew well on this medium were then screened for cellulase production in the following medium :

| | |
|---|---|
| Ammonium sulphate | 3 g |
| Sodium chloride | 500 mg |
| Magnesium sulphate ($7H_2O$) | 800 mg |
| Ferrous sulphate ($7H_2O$) | 10 mg |
| Dipotassium hydrogen phosphate | 1 g |
| Yeast extract | 1 g |
| Distilled water to 1 L | |

100 ml amounts was distributed into 250 mls Erlenmeyer flasks and 50 mg of absorbant cotton wool added. The flasks were autoclaved at 15 lbs per sq. in for 15 minutes. Culture filtrates were tested for cellulase activity at pH 4.8 after 5 days incubation at 30°C on an orbital shaker (320 rev/min).

Tannase production: Aspergillus niger M1 and Trichoderma koningii M223 were tested for the production of tannase in the following medium (Yamada et al, 1968):

| | |
|---|---|
| Sodium nitrate | 2 g |
| Potassium dihydrogen phosphate | 1 g |
| Magnesium sulphate ($7H_2O$) | 0.5 g |
| Potassium chloride | 0.5 g |
| Tannic acid | 10 g |
| Distilled water to 1L | |

The medium was autoclaved at 15 lbs/sq. in for 15 minutes in 50 mls

amounts in 250 ml Erlenmeyer flasks. Inoculation was with a thick spore suspension ($10^8$ spores/ml) and the flasks were incubated at 30°C for 1 - 7 days on an orbital shaker, (320 revs/min). Culture filtrates were assayed for tannase, and the mycelium collected by filtration, washed and blended for 1 minute in ice-cold 0.1 M citrate-phosphate buffer pH 5.5 (Fullspeed in an Osterizer : John Oster, Wisconsin, U.S.A.) The resulting pulp was spun at 3000 g for 10 minutes and the supernatant used for estimation of intracellular tannase activity.

13. ISOLATION OF BASIDIOMYCETES

The ability to utilise both cellulose and lignin is an attribute typical of a group of Basidiomycetes - the white rot fungi (Kirk 1971). Attempts were made to isolate Basidiomycetes from soil which would grow on carob. Enrichment cultures consisting of 5 g of spent carob mixed with 20 g moist beechwood soil were incubated at 27°C for periods up to 6 months, keeping the soil moist by the judicious addition of distilled water. After 3 months some of the cultures were sterilised by three successive autoclavings and reinoculated with material from live cultures. In this way, it was hoped to discourage fungi which grow on the readily available fractions of the carob which were assumed utilised after 3 months, and encourage fungi which could utilise the more resistant carob fractions. Material from the enrichment cultures were plated out on selective media and fungi which grew examined microscopically for typical Basidiomycete morphology (e.g. absence of spores and presence of clamp connections).

Selective medium (Hunt and Cobb, 1971)

| | |
|---|---|
| Benomyl | 8µg/ml |
| Dichloran (2 dichloro-4-nitroaniline) | 8µg/ml |
| Phenol | 50µg/ml |

Base - potato dextrose agar (Oxoid)

The inhibitors were made up in 50% ethanol at 200 times the working strength, sterilised by membrane filtration, and 1 ml added per 200 mls hot medium.

Selective medium (Russel, 1956)

| | |
|---|---|
| Malt extract | 3 g |
| Mycological peptone | 0.5 g |
| Agar | 2.5 g |
| o-phenyl phenol (o.p.p.) | 6 mg |
| Distilled water to 100 mls | |

The medium without the o.p.p. was sterilised at 10 lbs/sq. in for 10 minutes and the o.p.p. (sterilised membrane filtration) added from a stock solution containing 0.6 g/100 ml 50% ethanol. The pH of the medium was lowered to pH 5.5 with sterile lactic acid which had been sterilised by membrane filtration.

14. CAROB

All carob samples were supplied by Tate & Lyle Ltd. Kibbled carob was supplied as broken carob pods in pieces 0.5 - 2.0 cms long. The moisture content was determined by the method of Dean and Starke on 30 g carob samples heated for 3 days in

toluene (Dean and Starke, 1920).

Spent carob was prepared by Tate & Lyle in their laboratories remaining after a two-cycle hot water extraction of carob pods. The syrup is drawn off and more syrup is removed from the pods by pressure. The extracted carob is known as spent carob. Spent carob was supplied deep frozen and was stored at - 20°C. It was freeze-dried before use.

15. GROWTH EXPERIMENTS

A. SPENT CAROB AS THE SUBSTRATE-SLURRY FERMENTATIONS

All growth experiments using spent carob were in triplicate. Spent carob was suspended in a number of media to make it 2% w/v.

(i) CZ medium : a medium based on the Czapek-Dox forumla used : Nitrogen source : one of the following salts to give 400 mg N/L : ammonium sulphate, sodium nitrate, di-ammonium hydrogen phosphate, urea

| | |
|---|---|
| Sodium chloride | 500 mg |
| Magnesium sulphate ($7H_2O$) | 500 mg |
| Ferrous sulphate ($7H_2O$) | 10 mg |
| dipotassium hydrogen phosphate | 1 g |
| Distilled water to | 1 L |

autoclaved 15 lbs/sq. in for 15 minutes.

(ii) TL medium : as used by Tate & Lyle for the submerged culture of A. niger M1.

| | |
|---|---|
| Ammonium sulphate | 3.5 g |
| Sodium dihydrogen phosphate | 1.0 g |
| Distilled water to 1 L | |

autoclaved to 15 lbs/ sq. in for 15 minutes.

(iii) No nitrogen : as CZ but without the nitrogen source.

(iv) Unsupplemented : the carob was suspended in distilled water only.

Flasks containing 50 mls of medium were inoculated with 1-2 x $10^7$ spores produced from malt extract agar slants (Oxoid) and suspended in 0.05% Tween 80. Incubation of the flasks was carried out on an orbital shaker (320 rev/min) at 30-32$^{o}$C. Flasks were harvested by filtration on a Buchner funnel on a preweighed filter paper and the pH of the filtrate measured. The residue was then washed three times with 10 mls of distilled water and the filtrates diluted to 100 mls and assayed for total sugars and ammonia. The washed residue and filter paper were freeze-dried and reweighed. The yield of fungus and unused carob could thus be obtained. Kjeldhal nitrogen estimates, and in some instances Updegraff cellulose determinations, were made on the residue after separation from the filter paper. (This separation was easily performed when the carob was freeze-dried, but with difficulty if oven-dried). Each experiment contained uninoculated flasks as

controls which were sampled together with test (inoculated) flasks.

B. SPENT CAROB AS THE SUESTRATE - SOLID SUBSTRATE FERMENTATIONS

11 mls of TL medium containing 2.3% ammonium sulphate and 0.2% sodium dihydrogen phosphate were added to 5 g of ground freeze-dried carob ( 10 mesh). This produced a substrate containing about 70% moisture and with 10 mgN/g carob, the quantity of nitrogen used by M1 in 2% spent carob slurry experiments. The addition of nitrogen in higher concentrations was avoided to eliminate any toxicity problems (as encountered with cassava fermentations using very high nitrogen to carbon ratios, Young, personal communication). The carob "crumble" was spread evenly in 5 x 5 cm galvanised wire trays (a smaller version of those used by Stanton and Wallbridge (1969). These trays were then autoclaved in glass crystallising dishes at 15 lbs/sq. in for 15 minutes. After cooling, the substrate was evenly seeded by pipetting 2 mls of a spore suspension containing 2 - 3 x $10^7$ spores/ml evenly over the surface and incubated in a dessicator at 30-32$^{o}$C through which warm, moist air was passed. The air was humidified to 95-97% saturation, as used by TPI, (Young, personal communication) by passing it through saturated potassium sulphate (O'Brien, 1948); but later through 5% sulphuric acid and

a vapour trap of glass wool, since crystals of potassium sulphate formed on the spargers, blocking the air flow (Wilson, 1921). Cultures were in duplicate. Harvesting was performed by blending the fermented substrate with 40 ml. of 0.1% Tween 80 for 30 seconds at full speed in an osteriser (J. Oster, Wisconsin, U.S.A.). 0.1% Tween 80 has been found to be more efficient than distilled water for enzyme solubilization (Mandels et al, 1971). The homogenate was then spun at 3,000 g for 10 minutes and the supernatant decanted. The sediment was washed in a further 40 mls of 0.1% Tween 80 and respun. The supernatants were combined, made up to 100 mls, and cleared by spinning at 27,000 g for 15 minutes. The fermented carob was washed onto a pre-weighed filter paper and freeze dried, after which it was used for Kjeldhal nitrogen determination. The supernatant obtained after high speed centrifugation was assayed for reducing sugars, total sugars, ammonia and the pH recorded. 95 mls of the supernatant were saturated with ammonium sulphate at room temperature, stirred for 1 hour and then centrifuged at 3,000 g for 15 minutes. The precipitate obtained was washed twice with saturated ammonium sulphate and resuspended in 10 mls (i.e. one tenth of the original volume) of distilled water and dialysed overnight at $4^{o}$C against 4L of distilled water, to remove most of the ammonium sulphate. The resulting protein solution which

was then made up to 20 mls was largely free of reducing sugars by virtue of the ammonium sulphate precipitation step. It was then immediately assayed for cellulase, tannase and phenol-oxidase activity.

C. MODIFICATIONS OF SUBSTRATES

(i) Ball milling : ball milled (BM) carob was produced by ball-milling freeze-dried spent carob which had been ground to pass a 10 mesh sieve for 18 hours. BM carob was supplied as 2% w/v in TL medium.

(ii) Alkali swelling : two methods were used. The first (Pew and Weyna, 1962) used an alkali extraction of the spent carob, after which it was then washed, air dried and supplied at 2% w/v in TL medium. 10 g samples of carob were swollen in 200 mls of 2N sodium hydroxide for 30 minutes at 30°C. 200 mls of 2N acetic acid was then added and the mixture filtered on a sintered glass filter (porosity 1). Addition of the acid was found necessary to produce floculation of the carob which promoted rapid filtration. The material was then washed with distilled water until thepH of the filtrate remained unchanged. The carob was finally washed with acetone and air dried.

The second method of alkali swelling eliminated the washing procedure and subsequent loss of carob solubles (Doneffer et al, 1969). 1.0 g samples of freeze-dried carob were incubated with 20 mls of 2N sodium hydroxide for 30 minutes at 30°C. 20 ml of 2N acetic acid were then added,

followed by 10 mls of pentaple strength TL medium to give 2% carob in TL medium. Flasks were then autoclaved and inoculated in the normal manner.

D. KIBBLED CAROB AS THE SUBSTRATE - SLURRY CULTURES

100 g of kibbled carob were boiled for 1 minute with 400 mls of distilled water. The infusion, after it had cooled was blended in a Kenwood chef major at full speed for 1 minute, and the resultant slurry made up to 500 mls. Sodium dihydrogen phosphate was added to give a 0.1% solution and ammonium sulphate added to give concentrations of 0.35% to 2%.

The distribution of particle size was found in three different batches of slurried carob by wet-sieving the slurry and drying the sieves together with their fraction of carob to a constant weight at 105$^{o}$C. Aliquots of the 20% "stock" carob slurry were then diluted to 50 mls with the same salt solution as used for the preparation of the slurry and dispensed into 250 mls Erlenmeyer flasks to provide a range of carob slurries from 2-18% w/v. Vigorous stirring of the stock carob was essential during the dispensing procedure to ensure that a representative range of particle sizes reached each flask. The flasks were then autoclaved, inoculated and harvested as for the spent carob slurries. Experiments were performed with duplicate or triplicate flasks for each experimental

condition, and an equal number of uninoculated flasks were incubated as controls.

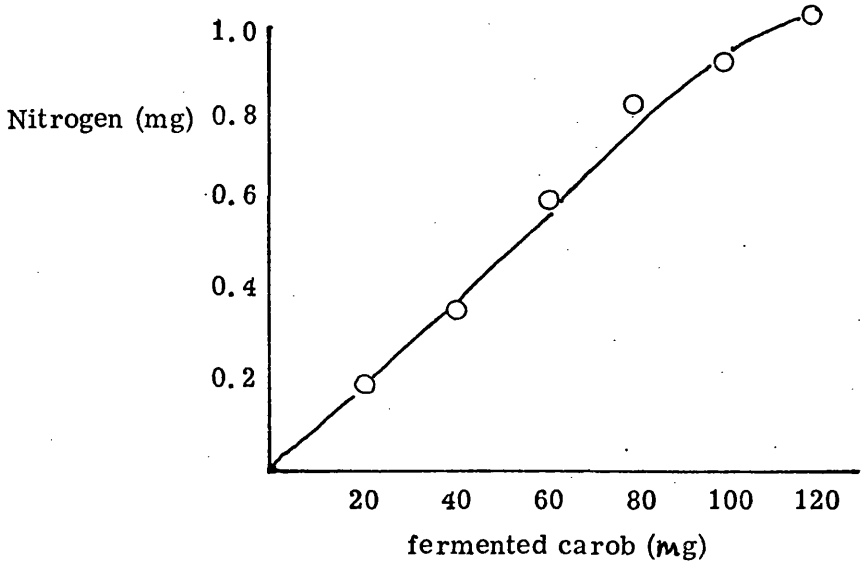
# RESULTS

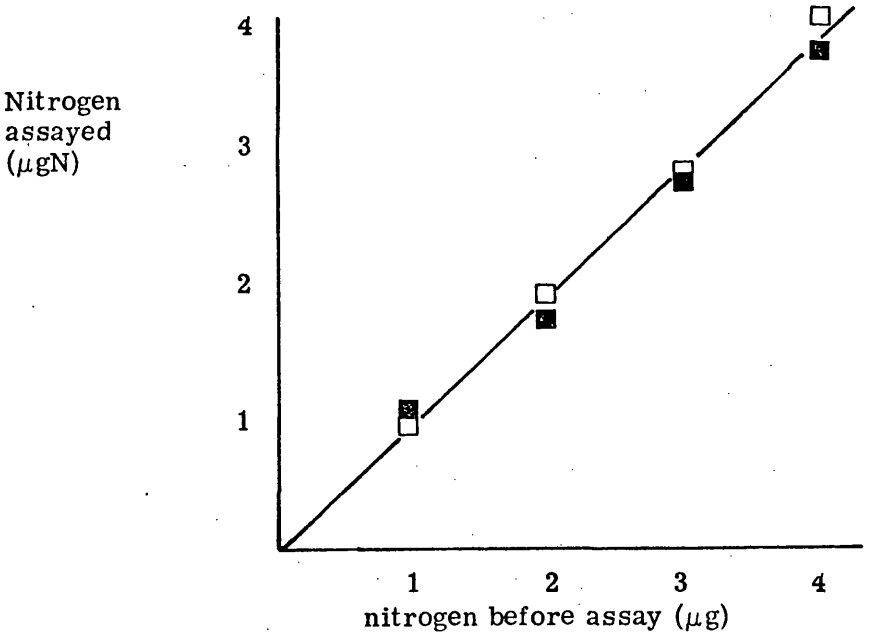
## DETERMINATION OF PROTEIN - SUITABILITY OF METHOD

The method of protein determination (Gørsuch et al, 1969) is based on the well-known Lowry procedure which suffers from interference by a number of substances, including phenols (Huang et al, 1971). Carob, being rich in tannins, proved a wholly unacceptable material for protein determination by this method - the high phenolic content producing estimates of more than 80% "protein" in fermented residues, an unlikely situation since the mycelium in the pure state contains only 20-30% protein (Tate & Lyle, 1971). Protein estimates based on Kjeldhal nitrogen x 6.25 gave more reliable results. The effect of sample size on nitrogen estimates is shown in figure 2. For routine purposes, samples size was adjusted to give a nitrogen content within the linear portion of the curve. Very little ammonia (and thus nitrogen) was lost during the procedure as shown in figure 3, which shows values for nitrogen determinations on ammonium sulphate samples with and without acid digestion. This method of protein estimation was soon superseded by the method using an alkali extraction, upon which nitrogen estimates were made. The protein in large carob samples (e.g. up to 1.2 gms carob) could be adequately extracted in 50 ml 1NaOH digests, since the relationship between sample size and estimated nitrogen content was linear (figure 4). Sample sizes were adjusted to give nitrogen contents within this linear range. Chitin was not solubilised by this method as indicated by the lack of detectable nitrogen in NaOH digests of chitin (figure 5). Also shown in

Figure 2. The effect of sample size on nitrogen estimation by the Kjeldhal technique on spent carob.

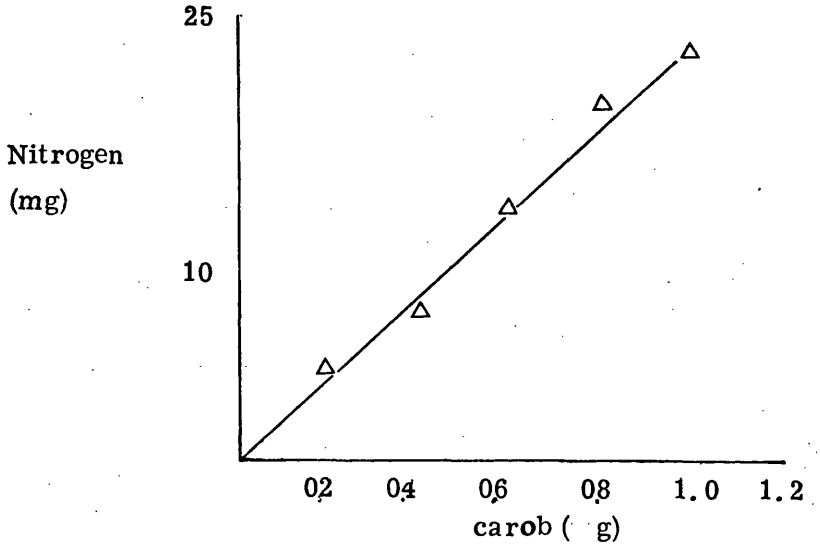
Figure 3. The effect of the Kjeldhal procedure on ammonium sulphate standards, before digestion □—□ ; after digestion ■–■ .


Nitrogen (mg)
1.0
0.8
0.6
0.4
0.2
20
40
60
80
100
120
fermented carob (mg)


Nitrogen assayed (μgN)
4
3
2
1
1
2
3
4
nitrogen before assay (μg)

F igure 4. The effect of sample size on nitrogen estimation by the Kjeldhal technique on sodium hydroxide extracts of spent carob.

Figure 5. The suitability of using a sodium hydroxide extraction for Kjeldhal nitrogen estimation. Standard curve, bovine serum albumin △——△; chitin ▲——▲ .


25
Nitrogen
(mg)
10
02
04
06
08
1.0
1.2
carob ( g)

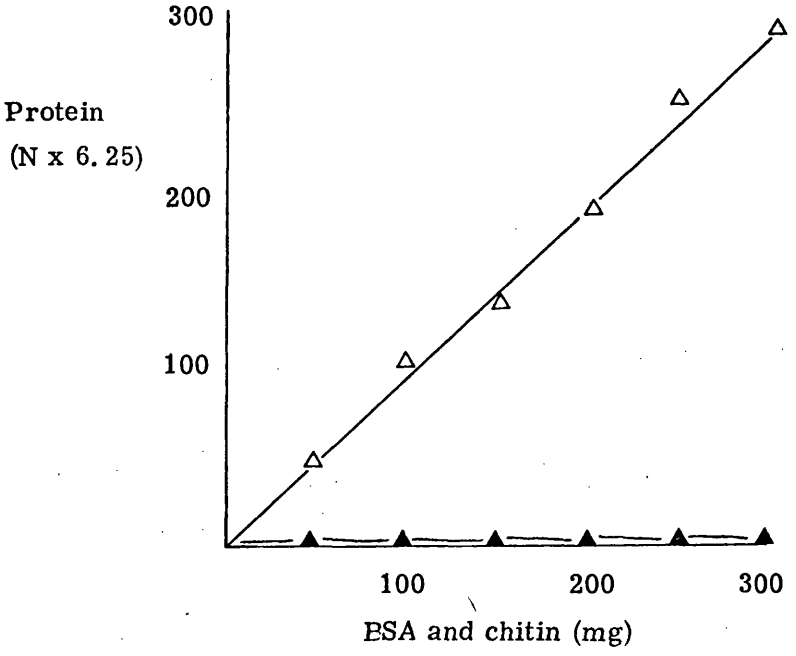

300
Protein
(N x 6.25)
200
100
100
200
300
BSA and chitin (mg)

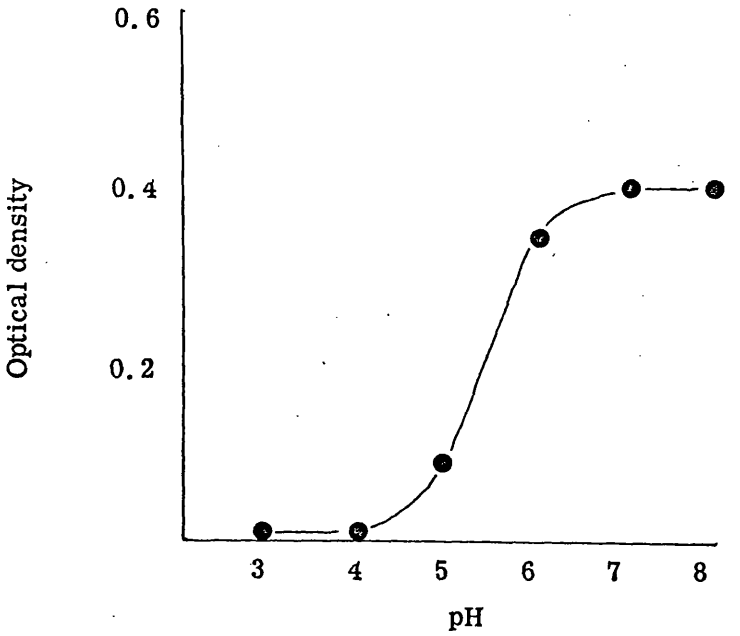
figure 5 is a bovine serum albumin standard curve showing that it contains 95% protein when subjected to this assay procedure. The two methods of protein determination (i.e. with and without an alkali extraction), were applied to a powdered freeze-dried sample of Aspergillus niger M1 mycelium supplied by Tate & Lyle Ltd. Quadruple determinations by each method yielded average protein contents of 41.0% and 35.1% in the original and modified Kjeldhal methods respectively. The difference could well be due to the fact that chitin is not estimated in the later method.

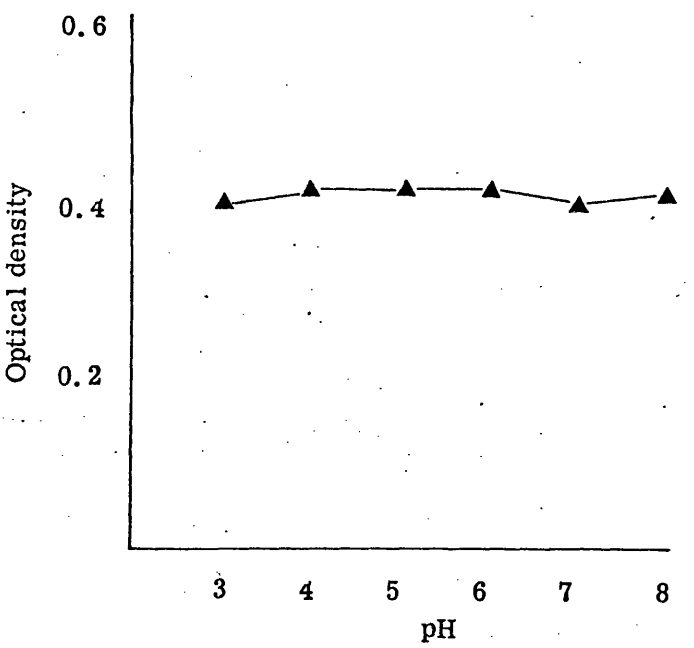
Cellulase determination

The principle of the cellulase assay was to estimate the amount of reducing sugar produced from incubating cellulase with cellulose (Mandels and Reese, 1964). Using 1 ml of enzyme-substrate reaction mixture and equal volumes of Somogyi and Nelson reagents (1:1:1), the colour produced was markedly dependent on pH of the reaction mixture (figure 6). The use of a 1:3:3 ratio improved the colour development over a wide pH range and this ratio was adhered to for subsequent glucose determinations (figure 7). The pH profiles of 3 fungi which produced cellulase in the cotton wool medium are shown in figure 8. It is apparent that Trichoderma koningii M223 cellulase had a much narrower pH range than the others, although it had the greatest activity of the 3 fungi tested. In order to ascertain that the assay conditions were not limiting during the 1 hour incubation period employed, samples of cotton wool were

Figure 6. The effect of pH on the Nelson-Somogyi method for identical glucose standards, with a 1:1:1 ratio of sample : Somogyi reagent : Nelson reagent.

Figure 7. The effect of pH on the Nelson-Somogyi method for identical glucose standards, with a 1:3:3 ratio of sample : Somogyi reagent : Nelson reagent.


0.6
0.4
0.2
Optical density
3
4
5
6
7
8
pH


0.6
0.4
0.2
Optical density
3
4
5
6
7
8
pH

incubated for 0-4 hours with the most active cellulase preparation (M223). A linear response was obtained indicating that the amount of reducing sugar produced is proportional to time, for at least a 4 hour incubation period (figure 9a). The amount of sugar produced was also directly proportional to the cellulase concentration (figure 9b). A milliunit of cellulase activity was defined as that amount of enzyme which produced 1mμM of glucose/min at 50°C from 50 mg cotton at pH 4.8 (International Union of Biochemistry, 1965).

ESTIMATION OF TANNASE

The estimation of tannase by the method of Iibuchi et al, (1967) proved unsuccessful. No enzymic activity towards tannin could be detected, even when M1 was grown with tannic acid as the sole carbon source, as in Yamada's medium (Yamada, et al, 1968). Since, in this medium, luxuriant growth was visible after 3 days, a tannase must have been produced - either intracellularly or extracellularly for the conversion of tannin into cell substance. However, increasing the volume of enzyme used in the routine mixture gave excellent results. A pH profile of cell-free culture filtrates from Aspergillus niger M1 grown in Yamada's medium shows a broad pH optimum around pH 5.5 (figure 10). Both time curves and enzyme concentration curves showed a linear response with respect to enzyme activity under

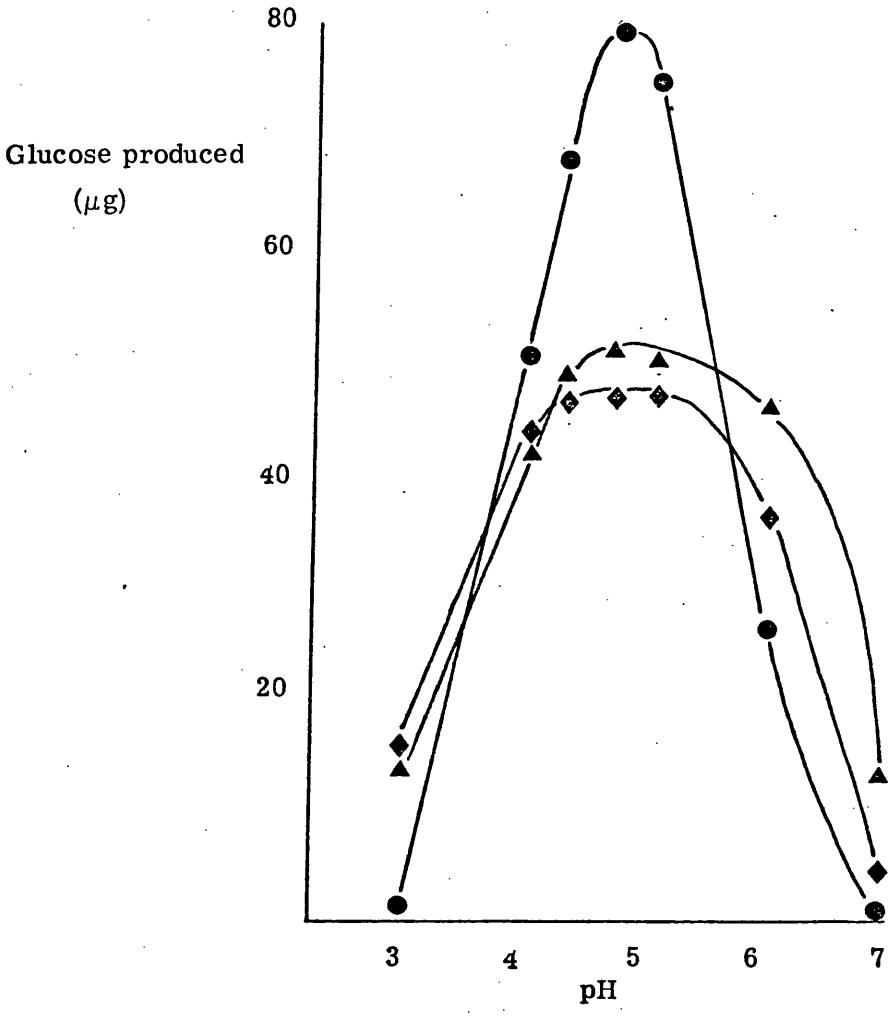
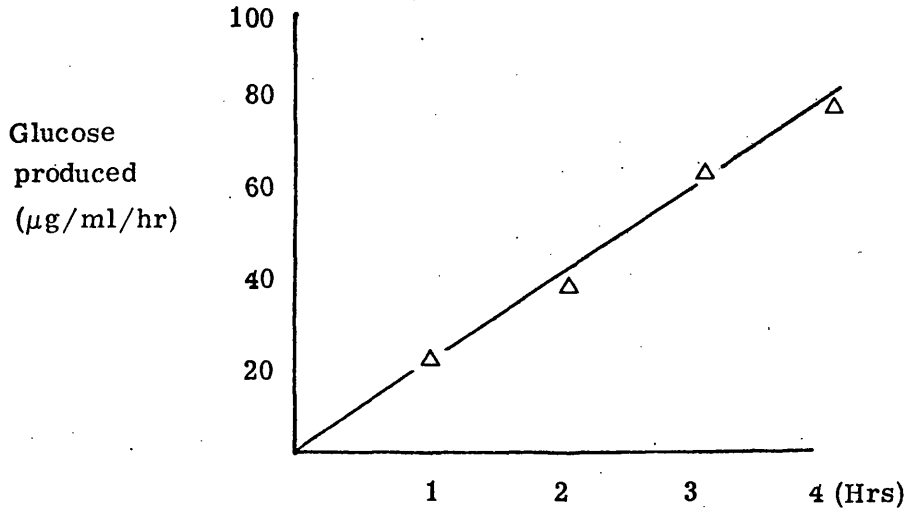
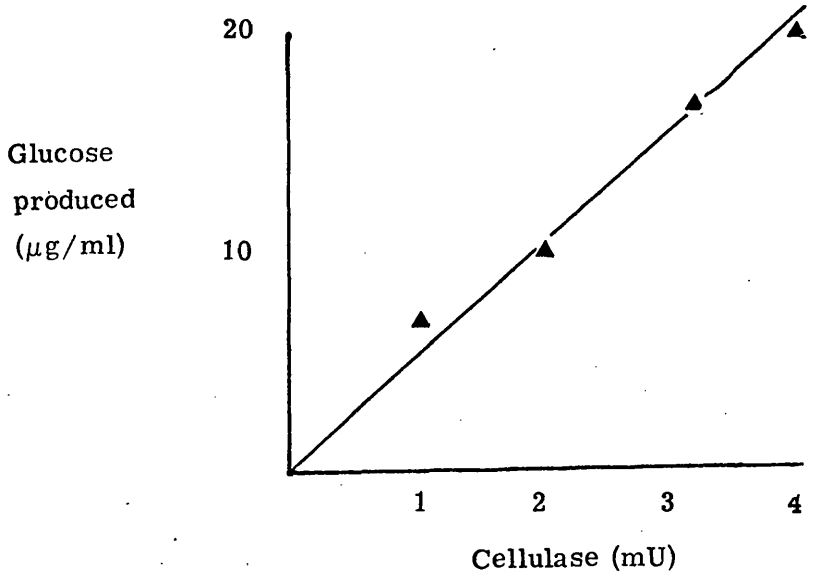



Figure 8. pH profiles of cellulases of fungi grown in cotton wool medium, ●—● ; Trichoderma koningii M223 ; ◆—◆, Trichoderma sp. M219; ▲—▲, Sporotrichum pruinosum M17.

Figure 9a. Activity of cellulase preparation from Trichoderma koningii M223 over a 4 hour incubation period.

Figure 9b. The relationship between cellulase concentration and enzymic activity of a cellulase preparation from Trichoderma koningii M223.


Glucose
produced
(μg/ml/hr)
100
80
60
40
20
1
2
3
4 (Hrs)


Glucose
produced
(μg/ml)
20
10
1
2
3
4
Cellulase (mU)

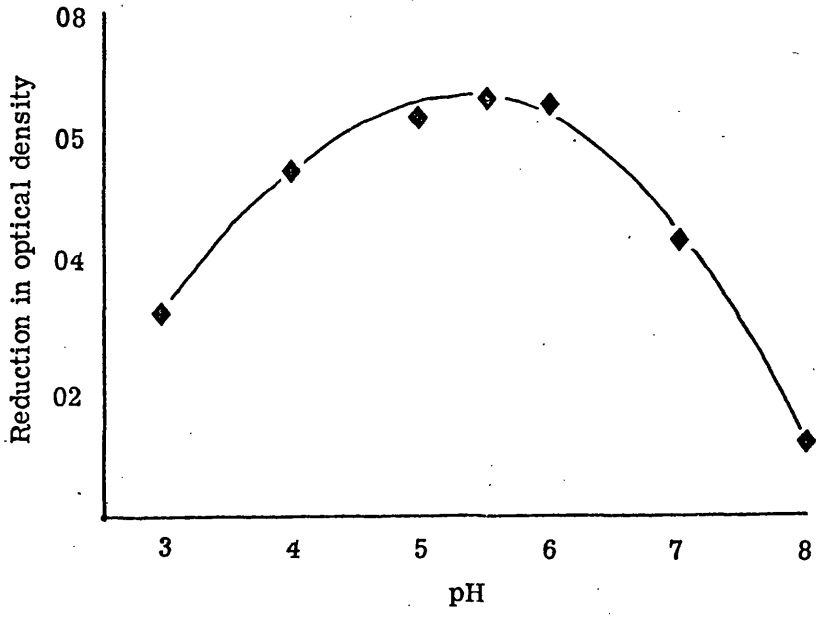



Figure 10. pH profile of tannase from Aspergillus niger M1 grown in a medium with tannic acid as the sole carbon source.

the conditions employed (figures 11a and b). Thus the enzyme assay could be used satisfactorily for monitoring tannase production in fermentations. The concentration of extracellular tannase was three times the intracellular tannase concentration after 3 days incubation in the tannic acid medium. A unit of enzyme activity was defined as that amount of enzyme which hydrolysed 1$\mu$M of the ester bond in tannic acid in one minute (Ibuchi et al, 1967).

PHENOL OXIDASE

None of the fungi tested (Aspergillus niger M1 and the selected cellulolytic fungi) produced phenol oxidase as detected by this method. Fungi were grown in 2% spent carob TL medium.

UPDEGRAFF TECHNIQUE FOR CELLULOSE ESTIMATION

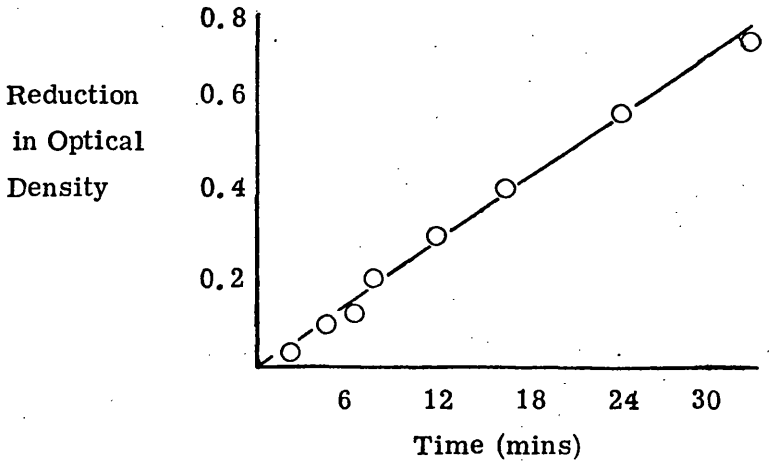
This assay produced consistent results and was a useful and rapid method of cellulose estimation. A number of substrates were tested for their suitability as standards, viz. Whatman cellulose powder, filter paper and ball-milled filter paper (table 3).

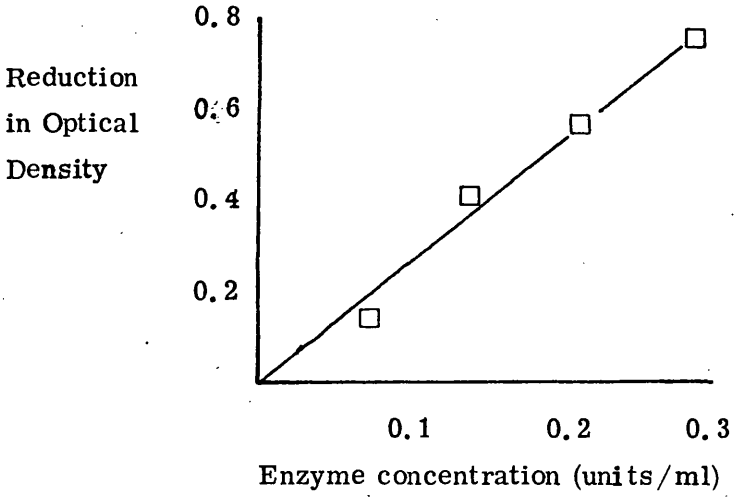
| Material | % cellulose |
|---|---|
| Whatman cellulose powder | 98.0 |
| Filter paper | 97.2 |
| Ball milled filter paper | 97.4 |
| Spent carob | 14.4 |
| Kibbled carob | 5.2 |

Table 3: Cellulose contents of cellulose and carob samples as determined by the Updegraff technique.

Figure 11a. Tannase activity of a culture filtrate of Aspergillus niger M1 grown in a medium with tannic acid as the sole carbon source.

Figure 11b. The relationship between tannase concentration and enzymic activity of a culture filtrate of Aspergillus niger M1 grown in a medium with tannic acid as the sole carbon source.


Reduction in Optical Density
0.8
0.6
0.4
0.2
6
12
18
24
30
Time (mins)


Reduction in Optical Density
0.8
0.6
0.4
0.2
0.1
0.2
0.3
Enzyme concentration (units/ml)

| | CAROB SAMPLE | | |
|---|---|---|---|
| FRACTION | KIBBLED | SPENT | |
| | | less than 10 mesh | ball-milled |
| Ethanol extractables | 61.8 | 11.3 | 18.4 |
| Water solubles | 14.1 | 28.3 | 16.0 |
| "Lignin" | 14.0 | 34.9 | 44.0 |
| Hemicellulose A | 5.2 | 6.5 | 5.6 |
| Hemicellulose B | 1.4 | 4.6 | 3.5 |
| $\alpha$-Cellulose | 5.5 | 14.4 | 12.9 |

Table 4a: Gravimetric composition of carob, expressed as percentage of whole carob.

| | CAROB SAMPLE | | |
|---|---|---|---|
| FRACTION | KIBBLED | SPENT | |
| | | less than 10 mesh | ball-milled (18 hr) |
| Ethanol extractables | 56.0 | 11.3 | 14.3 |
| Water solubles | 5.1 | 11.6 | 11.5 |
| TOTAL | 61.1 | 22.9 | 25.8 |

Table 4b: Sugar removed during ethanol and water extraction, expressed as percentage of whole carob.

Ball milling did not effect a depolymerisation of the cellulose in the filter paper with the subsequent reduction of the apparent cellulose content. Other workers have shown that subjecting cellulosic materials to a milling process increases the amount of extractable reducing sugars, perhaps by physically depolymerising the cellulose molecules, or the less crystalline regions of it (Katz and Reese, 1968). Whatman cellulose powder was used routinely as a standard since it was the most convenient form of cellulose to use. The cellulose content of kibbled carob and spent carob (both freeze dried) was estimated by this method (table 3). Washing the residue after acetic/nitric acid digestion with 5% alcohol as recommended by Crampton and Maynard (1938), but strangely not by Updegraff, greatly facilitated the centrifugation of the flocculant cellulose produced by the acid treatment. Three washes were found necessary to remove all the carob sugars, which if left with the carob residue, would be estimated as "cellulose" in the subsequent colourimetric assay.

THE FRACTIONATION OF CAROB BY THE METHOD OF JERMYN AND ISHERWOOD (1956)

The average weight losses incurred by the carob at each fractionation stage enable the gravimetric composition to be calculated (table 4a and b). It is possible to check the gravimetric analysis of Jermyn and Isherwood by estimating the cellulose content of material obtained from each stage of the analysis. If it is assumed that the final product of the Jermyn and

Isherwood fractionation, $\alpha$ cellulose, is chemically the same as the pure cellulose used for the standards in the Updegraff cellulose assay, then the cellulose content of material from the various stages of the Jermyn and Isherwood fractionation procedure estimated by the Updegraff technique, should agree with the cellulose content of the same fractions, back calculated from the $\alpha$ cellulose content of the whole carob and the respective weight losses produced at each fractionation stage. This comparison for spent carob is shown in table 5. As can be seen, the agreement between the two sets of figures is good, except for the $\alpha$ cellulose fraction, which is estimated to contain only 74% cellulose by the Updegraff technique. This could be explained by the fact that the $\alpha$ cellulose contains the same number of glucose residues/gram (i.e. of the same purity) but is of a less crystalline nature which might render it more soluble in the acetic-nitric reagent employed in the Updegraff technique. This reducion is crystallinity would have to occur during the preparation of $\alpha$-cellulose, since material prior to this step contains the expected amount of cellulose, as estimated by the Updegraff technique. Confirmation of this hypothesis was not furbished by experiments in which Whatman cellulose powder was subjected to the alkali extraction procedure used in the preparation of $\alpha$ cellulose from holocellulose. Material which had been alkali extracted was estimated to contain 101.4% cellulose, almost identical

to control batches which had undergone an equivalent distilled water extraction yielding 98% cellulose (using Whatman cellulase powder as a standard).

| | Cellulose content % | |
|---|---|---|
| Material | Calculated | Apparent |
| Whole spent carob | 14.4 | 13.9 |
| Ethanol ext. carob | 16.2 | 18.1 |
| Water ext. carob | 24.8 | 24.5 |
| Holocellulose | 54.2 | 54.4 |
| $\alpha$ cellulose | 100 | 73.7 |
| Hemicellulose | 0 | 1.9 |

Table 5. Comparison of apparent and calculated cellulose contents of spent carob at each fractionation stage of the Jermyn and Isherwood analysis.

One of the crucial stages of the Jermyn and Isherwood fractionation procedure is the delignification step. If the delignification procedure is carried on for too long, some of the cellulose will be solubilised, which (a) would produce an apparent high lignin content, since lignin is calculated on a percentage weight loss basis, and (b) lower the estimated cellulose content. In fact, the instigators of this delignification method have recommended that 1-2% lignin should be left after the "delignification"

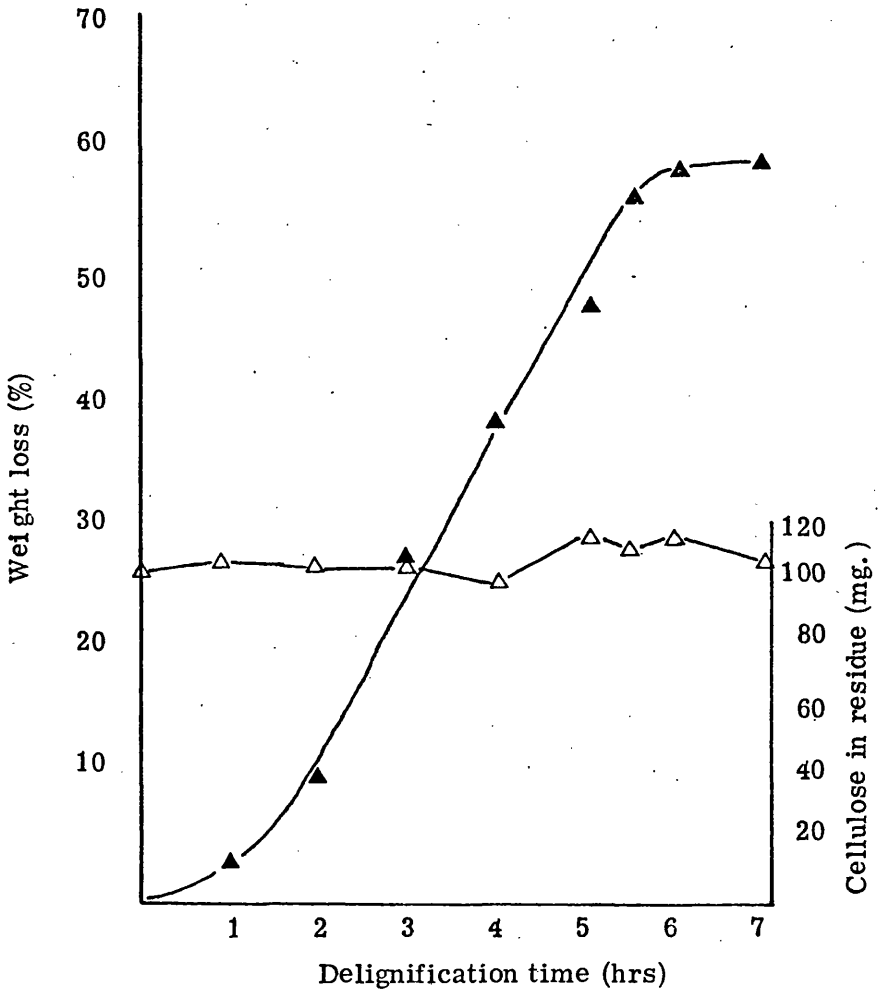



Figure 12. The effect of the duration of the delignification procedure on ethanol- and water-extracted carob. Weight loss, ▲—▲ ; cellulose, △—△.

procedure to ensure no cellulose is lost (Wise et al, 1946). To investigate the delignification process, duplicate samples of carob, which had been ethanol and water-extracted, were delignified under the usual experimental conditions described in the Materials and Methods, and sampled at intervals for 7 hours. The weight losses incurred by the carob and the cellulose content of the residue during the delignification procedure is shown in figure 12. It is apparent that no cellulose was lost during the delignification procedure even after the steady state (maximum weight loss) had been reached.

THE ESTIMATION OF TANNINS IN CAROB

Following the method for tannin estimation as laid down by the AOAC (1965 a), the tannin contents of kibbled and spent carob were 1.3% and 2.2% respectively. The tannin content of carob fermented by Aspergillus niger M1 determined by the AOAC and the modification using a colourimetric method for phenol estimation (Swain and Hillis, 1959) is shown in Table 6.

There is close agreement between the two methods which both show a 45% reduction in tannin content after fermentation. From Table 6 it is also evident that there is more tannin in the carob residues from the un-inoculated control flasks (4%) than in previous estimates on spent or kibbled carob. (2.2% and 1.3% respectively). The difference

| | Control (no inoculation) | | Inoculated | |
|---|---|---|---|---|
| | AOAC | Swain & Hillis | AOAC | Swain & Hillis |
| % tannin | 3.9 | 4.0 | 1.4 | 1.6 |
| Tannin/flask (mg) | 80 | 82 | 47 | 54 |

Table 6: Tannin contents of flasks with 10% kibbled carob and 1.25% ammonium sulphate after 3 days incubation.

between the kibbled carob and the carob residues from the control flasks is that the latter had undergone an autoclaving treatment and the soluble material removed by filtration. Therefore, it might be expected that carob from the control flasks would contain less tannin than native kibbled carob, since it had undergone an extraction process (autoclaving). Indeed, the filtrate had assumed a brown colour, probably indicative of tannins solubilised from the carob. Hence the effect of autoclaving the carob was studied more closely.

Increasing the number of hot water extractions in the AOAC procedure from one to three, exhaustively extracted the carob and increased the apparent tannin content (Table 7 a). However, if the first hot water extraction was replaced by a single autoclaving step (just as the un-inoculated control flasks had undergone) and then extracted with hot water, twice as much tannin was extracted. Further, spent carob which had undergone a hot water treatment by Tate & Lyle during

its preparation from kibbled carob, yielded even more tannin than kibbled carob. Examination of Table 7 b showing the tannin extracted during autoclaving expressed as a percentage of the carob sample reveals that the autoclaving procedure itself does not remove much more tannin than a hot water extraction, but induces a greater yield of tannin in the subsequent hot water extractions.

## SCREENING OF CULTURES

### a) Ability to grow on carob agar

The cultures provided by Tate & Lyle were screened for their ability to grow on carob agar, without excessive spore production (table 8). Excessive spore production has been considered to render fermented 'cheeses' unpalatable in the cassava process (Stanton and Wallbridge, 1969), although this may not be relevant for carob feeds, intended for animal consumption. It was realised that the ability of a fungus to grow on spent carob agar may not necessarily reflect its ability to grow in a carob slurry or on carob paste (as a solid substrate fermentation) where for instance, the concentration of carob, and therefore tannin, might be higher. However, those fungi growing on 2% spent carob agar should prove the most promising strains for tests in solid and slurry fermentations.

| Material | TOTAL TANNINS %<br>AOAC method | Swain & Hillis method |
|---|---|---|
| Kibbled carob | 3.2 | 3.9 |
| Autoclaved kibbled carob | 6.2 | 6.5 |
| Spent carob | 5.4 | 5.0 |
| Autoclaved spent carob | 10.0 | 9.0 |

Table 7a: The tannin content of carob: the effect of autoclaving.

| Material | TANNIN %<br>AOAC method | Swain & Hillis method |
|---|---|---|
| Kibbled carob | 1.3 | 2.1 |
| Autoclaved kibbled carob | 2.2 | 2.4 |
| Spent carob | 2.1 | 1.8 |
| Autoclaved spent carob | 3.8 | 3.0 |

Table 7b: The tannin content of carob : tannin removed during the first extraction procedure (hot water or autoclaving) expressed as a percentage of starting material.

Table 8: Screening experiments of cultures grown on 2% spent carob agar after incubation for one week. Growth assessed visually : -, no growth; +, poor growth; ++, moderate growth; +++, luxuriant growth. An asterisk (*) denotes profuse spore production.

| Fungus | Code (Tate & Lyle) | Growth temp. (°C) | Growth on carob agar |
|---|---|---|---|
| Aspergillus niger | M1 | 30 | +++ |
| " terreus | M11 | 27 | +++(*) |
| " niveus | M20 | 40 | + |
| " versicolour | M81 | 27 | ++ |
| " glaucus | M82 | 27 | + |
| " glaucus-oryzae | M85 | 27 | ++ |
| " fumigatus | M226 | 27 | +++(*) |
| Penicillium funiculosum | M9 | 27 | + |
| " baarnense | M21 | 40 | ++ |
| " verniculatum | M23 | 40 | + |
| " lilacinium | M45 | 27 | ++ |
| " chrysogenum | M47 | 27 | ++ |
| " janthinellum | M48 | 27 | + |
| " thorii | M49 | 27 | + |
| chrysogenum ii67 | M188 | 27 | ++(*) |
| " sp. | M24 | 40 | ++(*) |
| Myrothecium verrucaria | M3 | 27 | + |
| " " | M145 | 27 | + |
| | M146 | | |
| " " | | 27 | + |
| Fusarium oxysporum | M4 | 27 | +++ |

| Fungus | Code (Tate & Lyle) | Growth temp. (°C) | Growth on carob agar |
|---|---|---|---|
| Trichoderma koningii | M5 | 27 | +++ |
| " " | M223 | 27 | +++ |
| " viride | M7 | 27 | ++ |
| " sp. | M219 | 27 | +++ |
| Chaetomium globosum | M8 | 27 | +++ |
| " indicum | M30 | 27 | ++ |
| " globosum | M111 | 27 | ++ |
| Sporotrichum pruinosum | M10 | 27 | +++ |
| " " | M17 | 25/40 | +++ |
| Trichothecium roseum | M11 | 27 | +++ |
| Thermoascus aurantiacus | M14 | 40 | - |
| Ceratocystis paradoxa | M27 | 27 | - |
| Schizophyllum commune | M29 | 27 | + |
| Cladosporium cladosporoides | M32 | 27 | +++(*) |
| " " | M95 | 27 | +++ |
| Coniphora cerebella | M40 | 25 | + |
| Rhizoctona sp. | M43 | 25 | + |
| Rhizopus stolonifera | M208 | 27 | ++ |
| " sp. | M205 | 27 | - |
| Spicaria elegans | M217 | 27 | +++ |
| Oidium aureum | M26 | 25 | - |

| Fungus | Code (Tate & Lyle) | Growth temp. ($^{o}C$) | Growth on carob agar |
|---|---|---|---|
| Mouldy pulp isolate 1 | M227 | 27 | +++ |
| " " " 2 | M228 | 27 | ++ |
| " " " 3 | M231 | 27 | ++ |

b) Ability to produce cellulase

Those fungi which grew well on carob were then screened for cellulase production in cotton wool medium. It was interesting to note that very little mycelial growth was evident in the flasks, irrespective of the amount of cellulase produced (table 8).

BASIDIOMYCETE ISOLATION

Unfortunately, both of the selective media tested failed to suppress the growth of lower fungi, in particular, Rhizopus sp, which swamped the plate after 4-5 days incubation. Even when Rhizopus-free plates were obtained from suitably diluted serial dilutions of the enrichment culture, no basidiomycetes grew. Other fungi which tolerated the inhibitors and consistently formed colonies were Pythium sp, Trichothecium sp and Trichoderma sp. It was noted that many of the fungi which did grow showed gross morphological derangement, especially Rhizopus sp which produced abundant chlamydospores on the medium of Huntley and Cobb.

GROWTH EXPERIMENTS - I. SPENT CAROB

Since Aspergillus niger M1 is already successfully used for carob syrup fermentation, it was decided to examine its potential for upgrading spent carob. The selected fungi from the screening tests were kept as a second choice since no toxicity trials have been done with them.

| Fungus | Visible breakdown of cotton wool | Cellulase mU/ml of culture filtrate |
|---|---|---|
| Fusarium oxysporum M4 | + | 0 |
| Trichoderma koningii M5 | + | 0 |
| Chaetomium globosum M8 | ++ | 0 |
| Sporotrichum pruinosum M10 | +++ | 0 |
| Aspergillus terreus M11 | - | 0 |
| Sporotrichum pruinosum M17 | +++ | 2.8 |
| Trichoderma sp. M219 | +++ | 2.6 |
| Trichoderma koningii M223 | +++ | 4.5 |
| Mouldy pulp isolate (Trichoderma sp.) M227 | - | 0 |

Table 9: Cellulolytic activity of 10 selected fungi. Slight degradation of cotton wool +; moderate degradation ++; extensive degradation +++; no breakdown -.

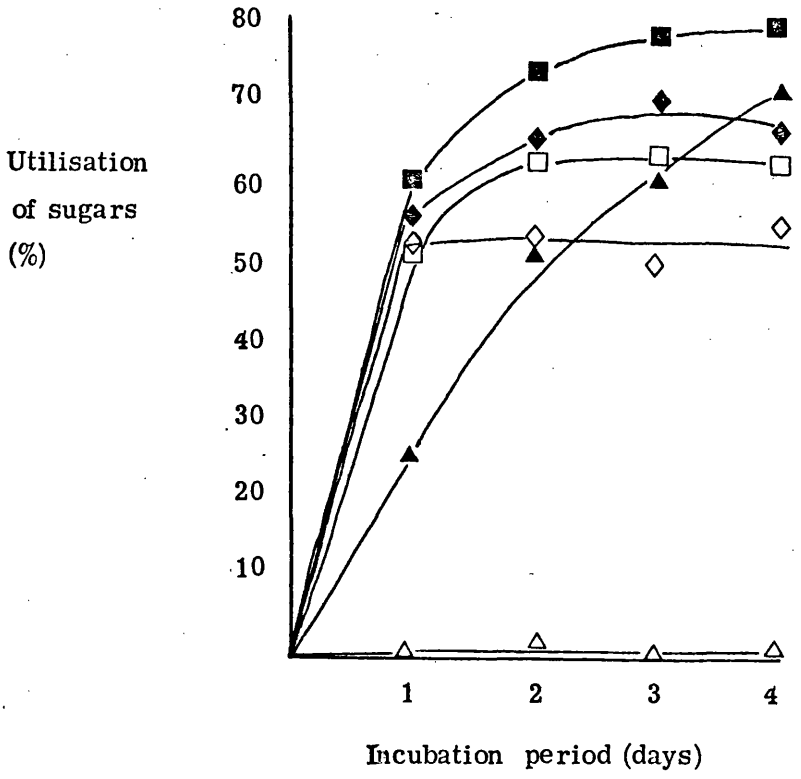



Figure 13. The effect of different nitrogen sources on the utilisation of sugars in 2% spent carob slurries. △—△ uninoculated control; ▲—▲ unsupplemented control; ■—■ ammonium sulphate; ◆—◆ sodium nitrate; ◇—◇ urea; □—□ diammonium hydrogen phosphate

(1) Effect of different nitrogen sources on the growth of Aspergillus niger in spent carob slurries.

From figure 13 it is evident that the incorporation of Czapek-Dox based medium into carob slurries increases the initial rates of utilisation of sugars although it does not increase the total utilisation at the end of the fermentation period. Comparing the nitrogen sources, ammonium sulphate gave the highest rate of sugar utilisation and percentage utilisation of sugars and also yielded some of the highest protein containing residues (figures 14a and b). All the nitrogen sources tested, with the exception of sodium nitrate, produced residues with similar protein contents. As might be expected, flasks which had no addition of a nitrogen source, although supporting good growth as witnessed by the utilisation of sugars, did not produce any increase in protein when determined by the Kjeldhal method - the total nitrogen of the solid residues after fermentation was the same as before fermentation. The pH of the Czapek-Dox based medium without a nitrogen source dropped steadily during growth of M1 (figure 15), whereas in those flasks with a Czapek-Dox based medium and a nitrogen source the pH did not reach such a low value. The complete media could thus be exerting a buffering effect or a medium without nitrogen is particularly inducive of a metabolism producing acidic products. The flasks with a nitrogen source all showed a substantial fall in pH after just one day's growth, whereupon further incubation did not substantially alter

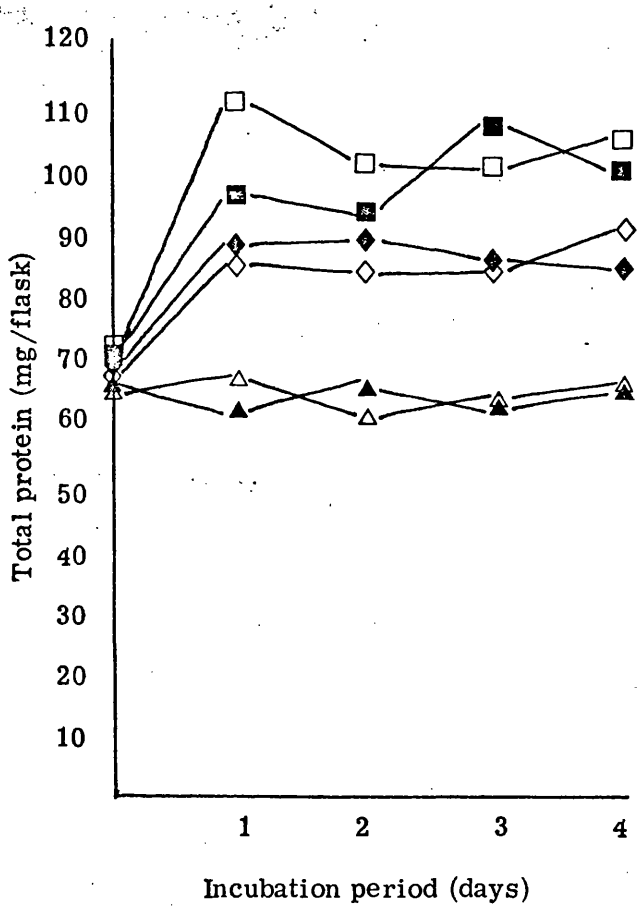
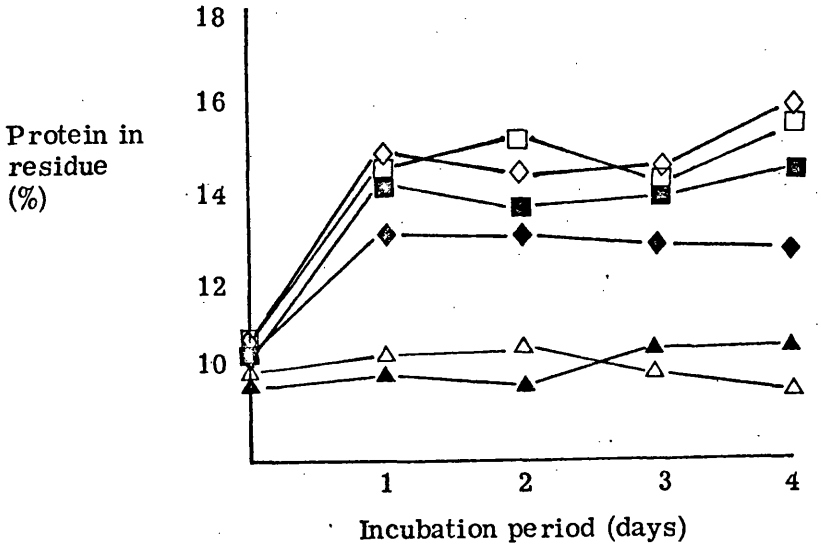
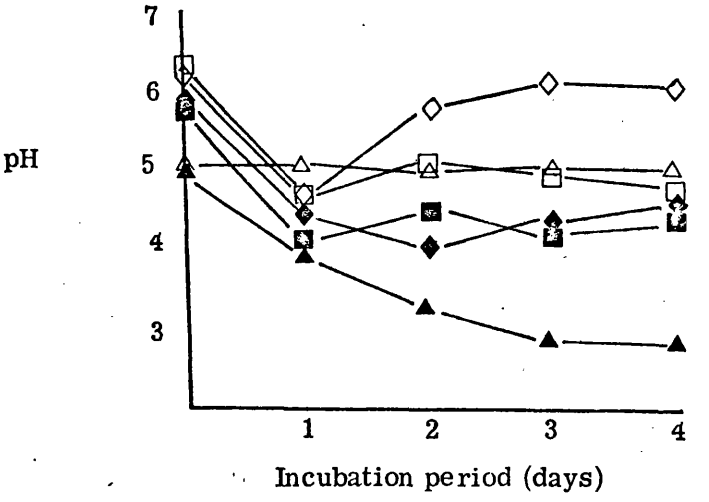



Figure 14a. Protein production in 2% spent carob slurries with different nitrogen sources. △—△ , uninoculated control; ▲—▲ unsupplemented control; ■—■ ammonium sulphate; ◆—◆ sodium nitrate; ◇—◇ urea; □—□ diammonium hydrogen phosphate.

**Figure 14b.** **Protein content of residues from 2% spent carob slurries with different nitrogen sources.** △—△ **uninoculated control;** ▲—▲ **unsupplemented control;** ■—■ **ammonium sulphate;** ◆—◆ **sodium nitrate;** ◇—◇ **urea;** □—□ diammonium hydrogen phosphate.

**Figure 15.** **The influence of different nitrogen sources on the pH of the culture filtrate of 2% spent carob slurries.** △—△ **uninoculated control,** ▲—▲ **unsupplemented control;** ■—■ **ammonium sulphate;** ◆—◆ **sodium nitrate;** ◇—◇ **urea** □—□ diammonium hydrogen phosphate.


Protein in residue (%)
18
16
14
12
10
1
2
3
4
Incubation period (days)


pH
7
6
5
4
3
1
2
3
4
Incubation period (days)

pH; except in flasks containing urea, where an increase in pH was observed.

Ammonium sulphate was chosen as the nitrogen source for subsequent investigations on the basis of its suitability from these trials and also because Tate & Lyle Ltd. already use it as a nitrogen source in TL medium for the fermentation of carob syrup. Thus only one nitrogen source would be needed for the complete fungal upgrading of carob. Later experiments used the complete Tate & Lyle medium which is used for the carob syrup fermentation.

(2) The effect of ammonium sulphate concentration on the spent carob slurry fermentation.

Having decided to use ammonium sulphate as the nitrogen source, the optimum concentration was sought. The effects of different ammonium sulphate concentrations on sugar consumption is shown in figure 16. The control flasks, containing Czapek-Dox based medium without a nitrogen source, initially increased the rate of utilisation of sugar over that in unsupplemented carob slurries, but did not increase the total sugar consumption. The addition of nitrogen in concentrations greater than 200 mgN/L had very little effect on the total utilisation of sugar. It is clear from figure 17 that the nitrogen was present in excess of requirement in all concentrations tested, since there was always nitrogen present in the culture filtrate after fermentation. Very

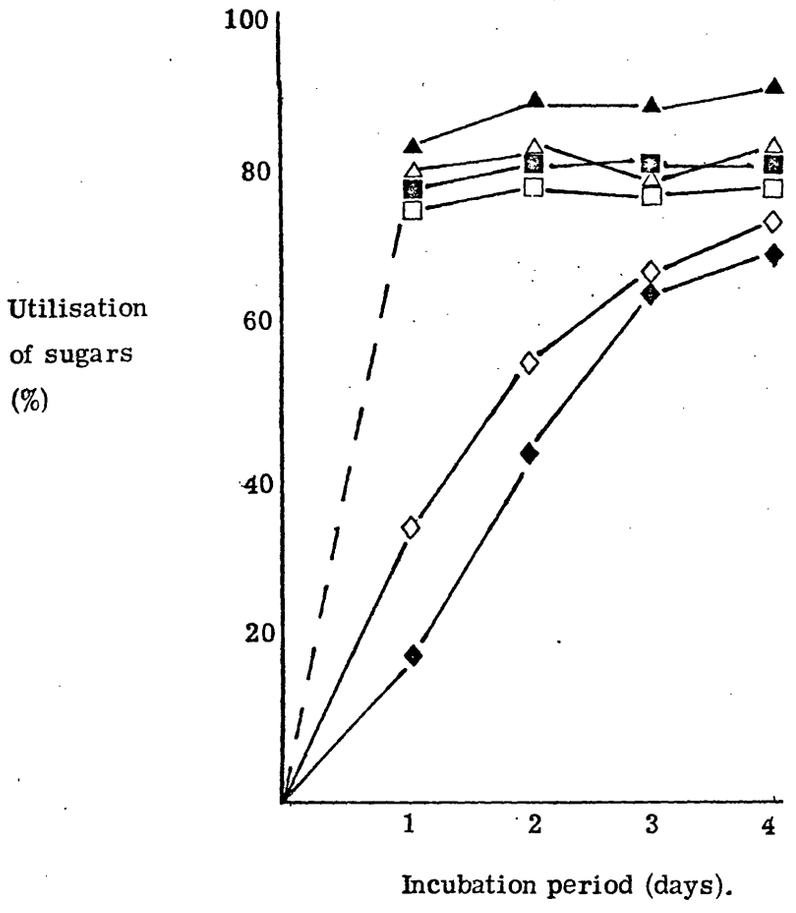



Figure 16. The effect of ammonium sulphate concentration on the utilisation of total sugars in 2% spent carob slurries ◆—◆ , control, no supplementation; ◇—◇ , control no nitrogen; □—□ 200 mg N/L; ◘—◘ 400 mgN/L; △—△ 600 mg N/L; ▲—▲ 800 mgN/L.

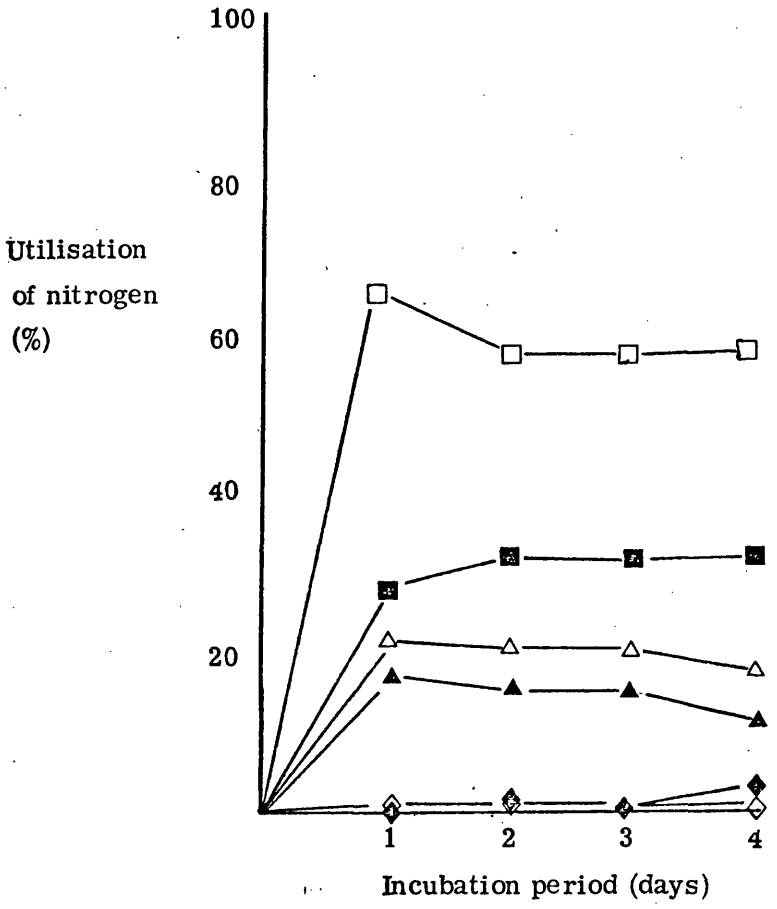



Figure 17. The effect of the ammonium sulphate concentration on the utilisation of ammonia-nitrogen in 2% spent carob slurries. ◆—◆ control, no supplements; ◇—◇ control, no nitrogen; □—□ 200mgN/L; ■—■ 400 mgN/L; △—△ 600 mgN/L, ▲—▲ 800 mgN/L.

little difference was observed in the protein contents of residues and the total protein synthesised (figures 18a and b). Thus an increase in the initial ammonium sulphate concentration does not produce a corresponding increase in the protein synthesised. This suggests that some environmental factor is limiting. The pH produced in carob slurries containing ammonium sulphate never fell below that produced in un-supplemented slurries (figure 19), so pH is unlikely to be inhibiting further protein synthesis. The possibility of substrate limitation was investigated.

(3) The effect of adding glucose to stationary-phase cultures.

To investigate the possibility of substrate starvation in spite of the presence of unused carob solids, as is evident from visual examinations of fermented cultures, glucose was added to fermented cultures after they had reached the stationary phase of growth.

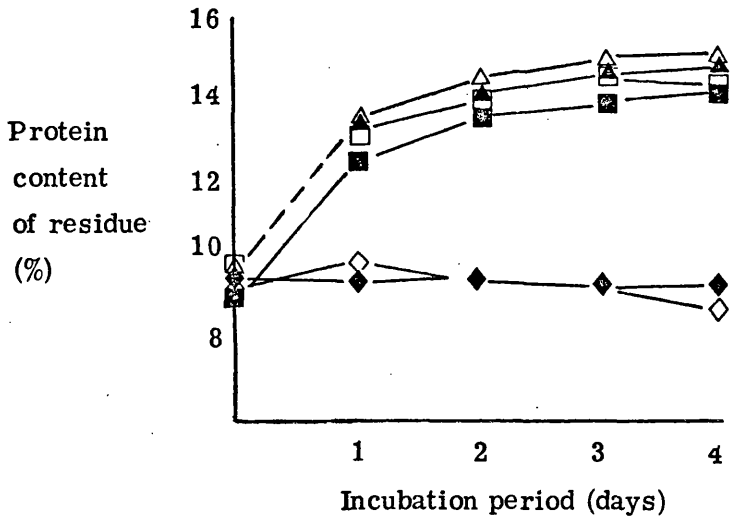
Three day old cultures, which were judged to be in the stationary phase by the preceding experiments, were divided into two batches. To one of these glucose was added, to the others, the controls, distilled water was added and both sets of flasks reincubated for a further two days. The results are shown in figure 20. It is immediately evident that flasks which had glucose added supported further growth, confirming the hypothesis that the fermentations had become carbohydrate limited.

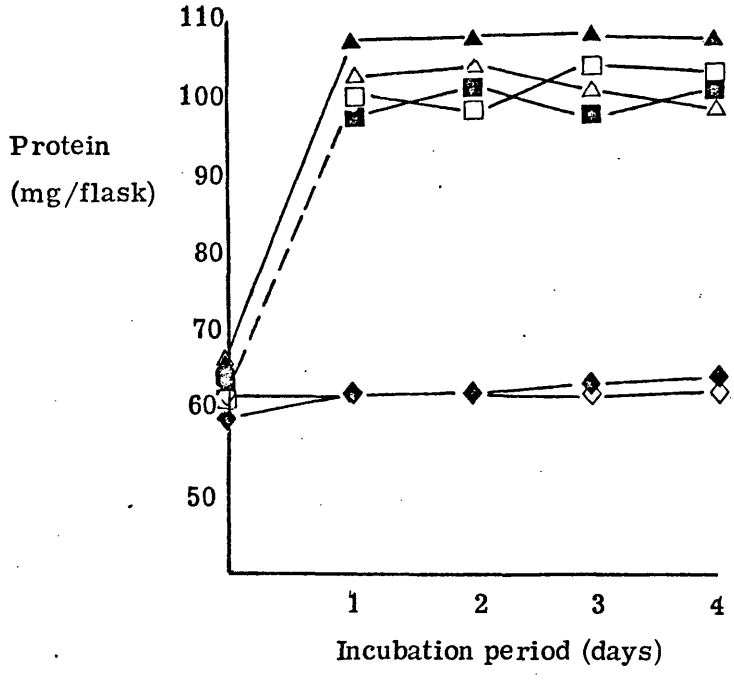
(4) The effect of modifying the substrate.

Procedures designed to render more of the carob

Figure 18a. The effect of the ammonium sulphate concentration on the protein content of the fermented residues in 2% spent carob slurries ◆—◆ control, no supplements; ◇—◇ control, no nitrogen; □—□ 200 mgN/L; ■—■ 400 mgN/L; △—△ 600 mgN/L; ▲—▲ 800 mgN/L.

Figure 18b. The effect of ammonium sulphate on the yield of total protein in 2% spent carob slurries ◆—◆ , control, no supplements; ◇—◇ control, no nitrogen; □—□ 200 mgN/L; ■—■ 400 mgN/L; △—△ 600 mgN/L; ▲—▲ 800 mgN/L.


Protein content of residue (%)
16
14
12
10
8
1
2
3
4
Incubation period (days)


Protein (mg/flask)
110
100
90
80
70
60
50
1
2
3
4
Incubation period (days)

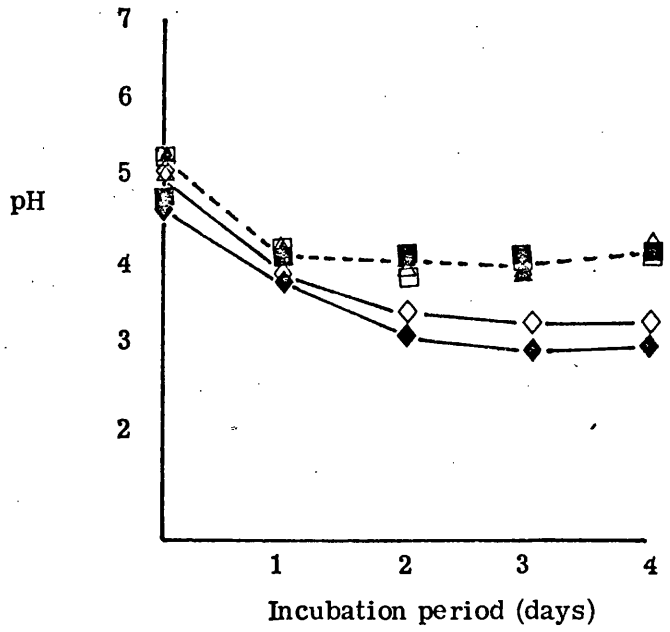



Figure 19. The effect of ammonium sulphate concentration on the pH of the culture filtrates in 2% spent carob slurries. ◆—◆ , control, no supplements; ◇—◇ , control, no nitrogen; □—□ 200 mgN/L; ■—■ 400 mgN/L; △—△ 600 mgN/L, ▲—▲ 800 mgN/L.

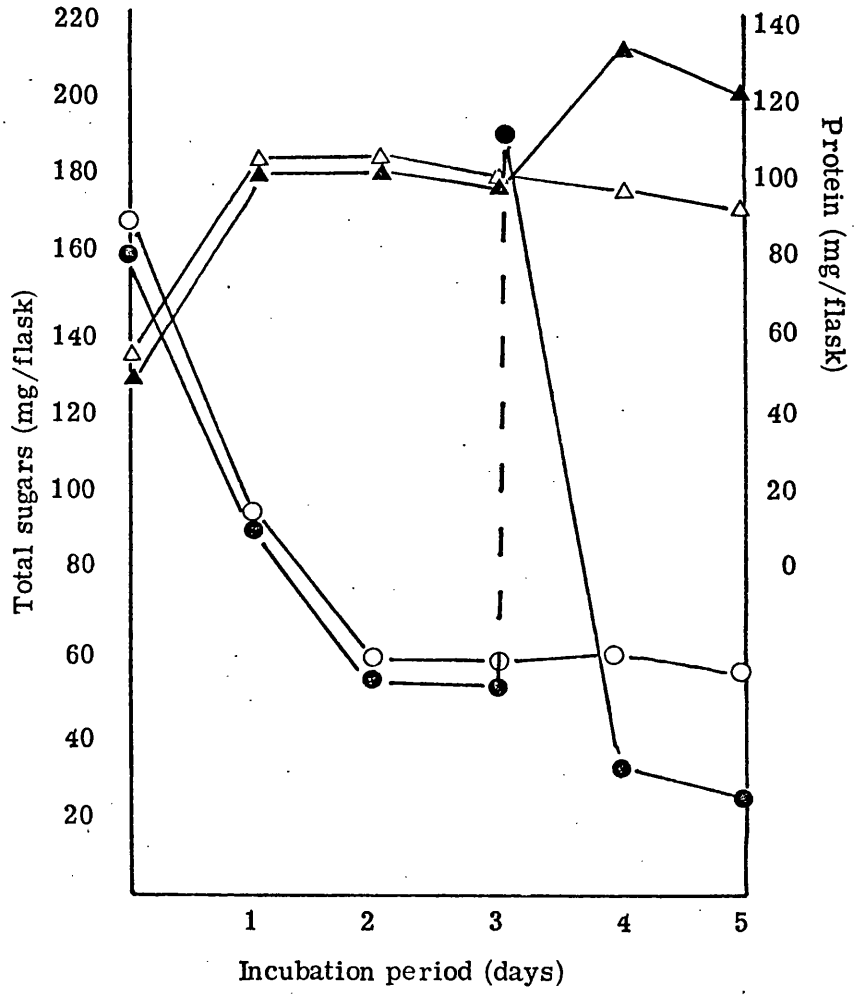



Figure 20. The effect of adding glucose to stationary phase cultures. Flasks containing 400 mgN/L, 2% spent carob. Glucose or distilled water added at 3 days. ●—● , total sugars and ▲—▲ , TCPL in flasks to which glucose was added. ○—○ , total sugars and △——△ , TCPL in flasks to which distilled water was added.

available to the fungus were tested. These procedures were ball-milling (18 hours) and alkali treatment with and without subsequent washing of the carob. The effects of the treatments on carob are shown in figures 21, a-d. Ball-milling the carob did not increase the sugars available to or utilised by the fungus, nor the protein synthesised, when compared with the usual <10 mesh ground carob. The carob which had been swollen in alkali and then neutralised without a washing process proved to be an unsuitable substrate for M1 by every parameter measured. However, the carob which had been alkali extracted and washed free of alkali did support growth after prolonged incubation. The protein content of this fermented residue (11.3%) was lower than that from control flasks containing carob which had not undergone an alkali extraction (14.8%).

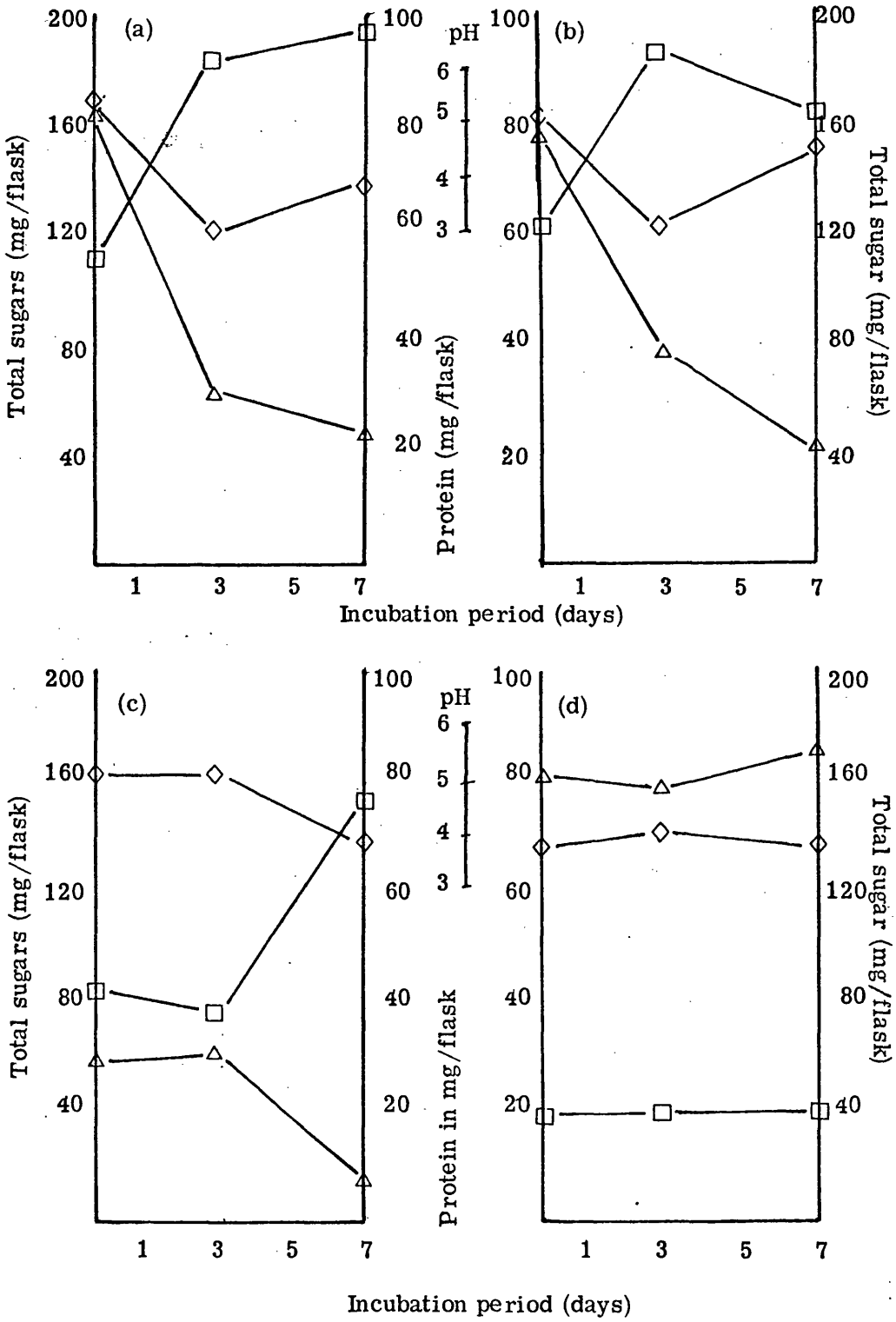
Both the alkali treatments solubilised some of the protein in the carob, producing the low protein contents observed at the start of fermentation.

(5) Solid substrate fermentations

The cellulolytic fungus chosen for further studies was Trichoderma koningii M223. Since this fungus would not grow in 2% spent carob slurries, except at the liquid/air interface, a solid substrate fermentation was tried.

Air borne contamination was found to be a problem in these solid substrate fermentations. This was substantially reduced if the air stream into the dessicator was passed through a suitable millipore filter; and if the dessicator itself was fumigated with formalin vapour overnight (by bubbling the air stream through

**Figures 21 a - d.** Modification of substrates (a) control, coarsely ground carob ( < 10 mesh) (b) 18 hour ball-milled carob (c) alkali swollen and washed carob (d) alkali swollen but not washed carob. △—△ total sugars; □—□ , total protein ◇—◇ , pH of culture filtrate.


(a)
(b)
(c)
(d)
Total sugars (mg/flask)
Total sugar (mg/flask)
Protein (mg/flask)
Protein in mg/flask
pH
Incubation period (days)

10% formalin), and then flushing with sterile air for 2 hours before use. The result of a typical solid substrate fermentation, using Trichoderma koningii M223 is shown in figure 22. Reducing sugar was consumed rapidly but the pH fell only slightly during the fermentation. The substrate assumed a green colour after 4 days of fermentation due to the prolific production of spores. Also, the substrate appeared to be thoroughly ramified by the fungus as judged by a visual examination of sections of the fermented carob. However, the protein content of the residue increased by only a small percentage, in spite of an apparently high utilisation of ammonia. No tannase could be detected during the course of the experiments but cellulase was present, in small amounts, towards the end of the fermentation period. The cellulose content of the residues, as determined by the Updegraff technique, remained unchanged. A few days prior to the production of cellulase, a non-cellulolytic enzyme was detected which produced glucose in the control tubes of the cellulase assay. Since the control tubes do not contain cellulose the enzyme could not be a cellulase. Therefore the culture filtrate concentrate contained both a soluble substrate and the enzyme responsible for glucose release. Precipitation of any tannin present by the addition of gelatin to enzyme concentrates, did not alter the ability of the supernatant to produce glucose in the absence of cellulose, suggesting that the sugar is not being produced through the action of tannase on tannic acid

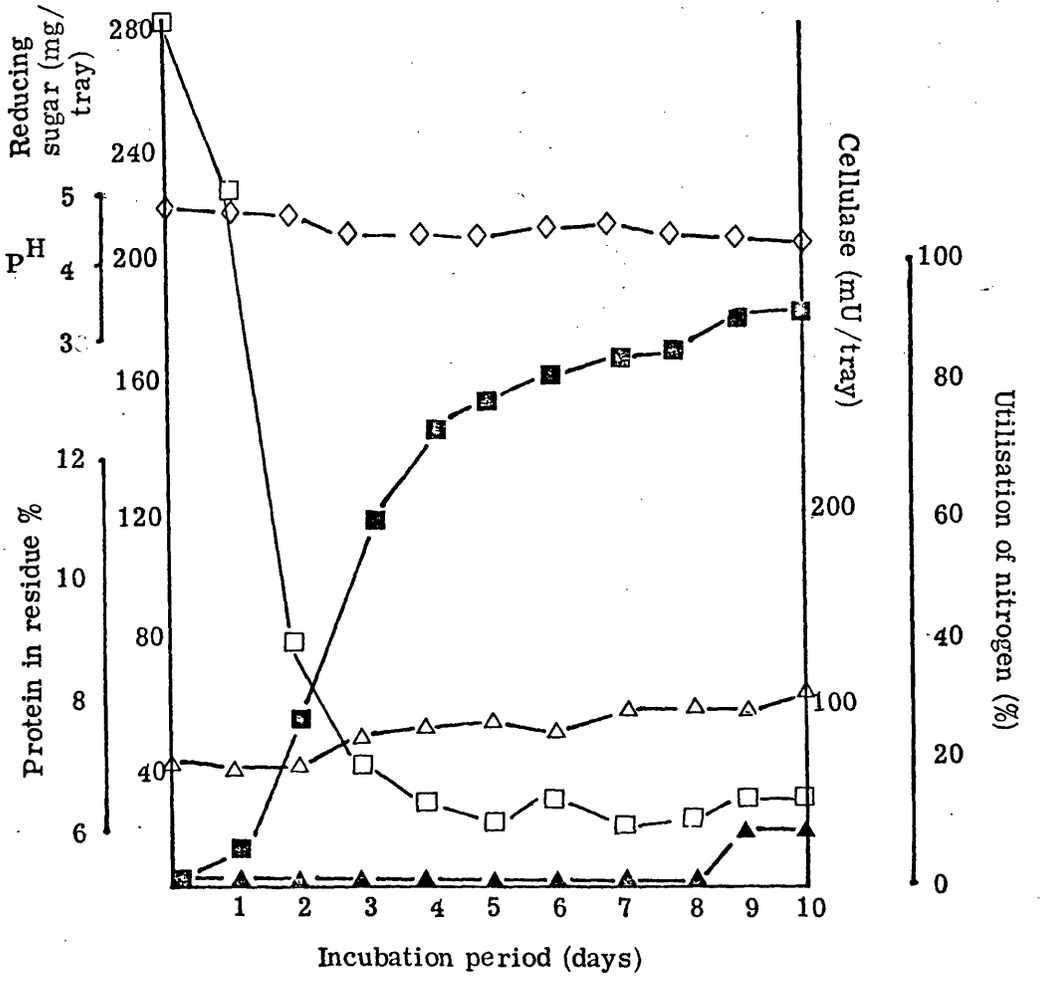



Figure 22. Solid substrate fermentation of spent carob by Trichoderma koningii M223. □—□ total sugars; ◇—◇ pH; ■—■ utilisation of ammonia; △—△ percentage protein of residue and ▲—▲ cellulase.

(to give glucose and digallic acid). It is evident that although reducing sugar concentrations are reduced to low values after 4 days incubation very little cellulase was produced. Cellulase could only be detected in extracts which had undergone concentration by ammonium sulphate precipitation, and from which most of the reducing sugar had been removed. The lack of cellulase production contrasts with the results of experiments using M223 in the cotton-wool medium used for screening cellulolytic fungi to which glucose had been added in amounts found in solid substrate fermentations (figure 23). Once the glucose was consumed, cellulase was produced. Interestingly, control flasks without this initial glucose concentration produced cellulase only after prolonged incubation, but in much larger amounts.

Growth experiments with kibbled carob

Preparation of substrate. The distribution of particle size obtained by the blending procedure described in the Methods section is shown in figure 24. Approximately 40% of the carob was reduced to particles greater than 1.7 mm. On visual examination, this fraction consisted almost entirely of the black outercoat of the pod. The remainder of the pod was very effectively reduced to particles of 0.5 mm or less. Seventy per cent of the kibbled carob solubilized by this treatment. Allowing for the fact that the kibbled carob was found to contain 15.0% moisture, the corrected weight loss

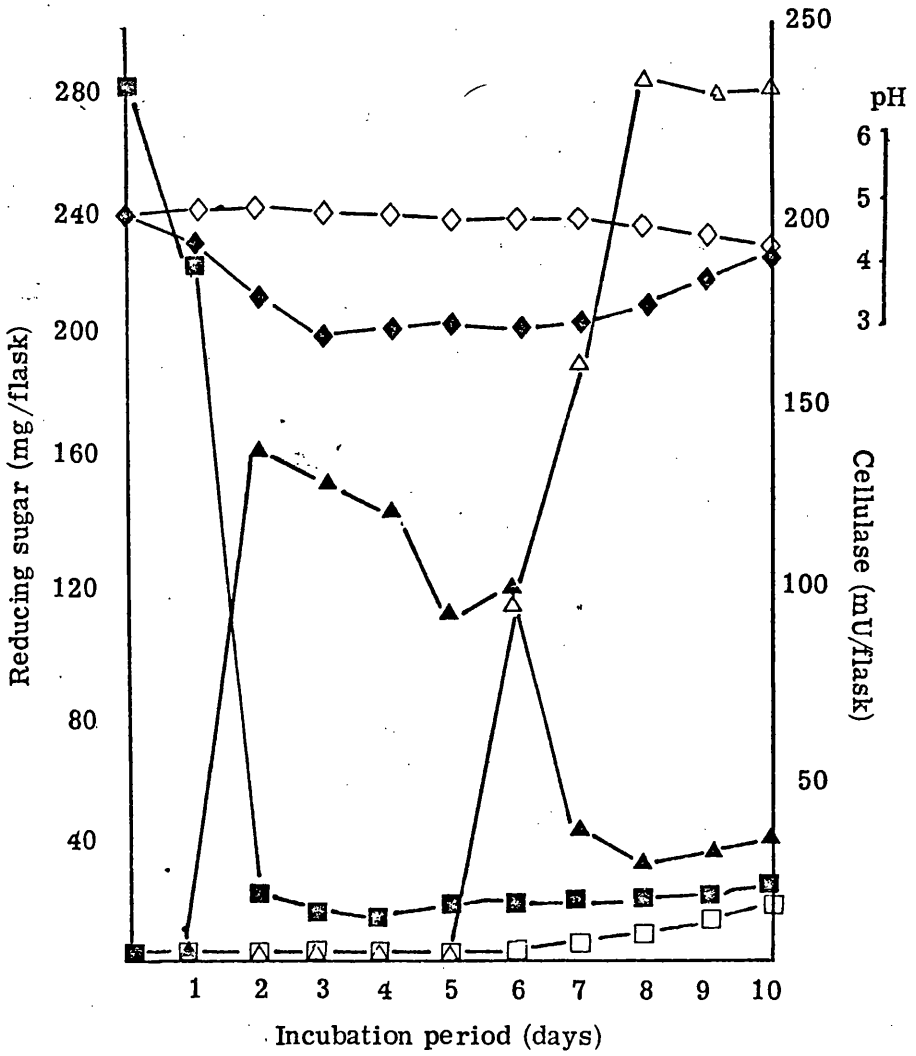



Figure 23. Growth of Trichoderma koningii M223 in cotton wool cultures, with and without the presence of glucose. No glucose: ▫–▫ reducing sugars; ◇—◇ pH; △—△ cellulase. With glucose: ■—■, reducing sugars; ◆—◆ pH; ▲—▲ cellulase.

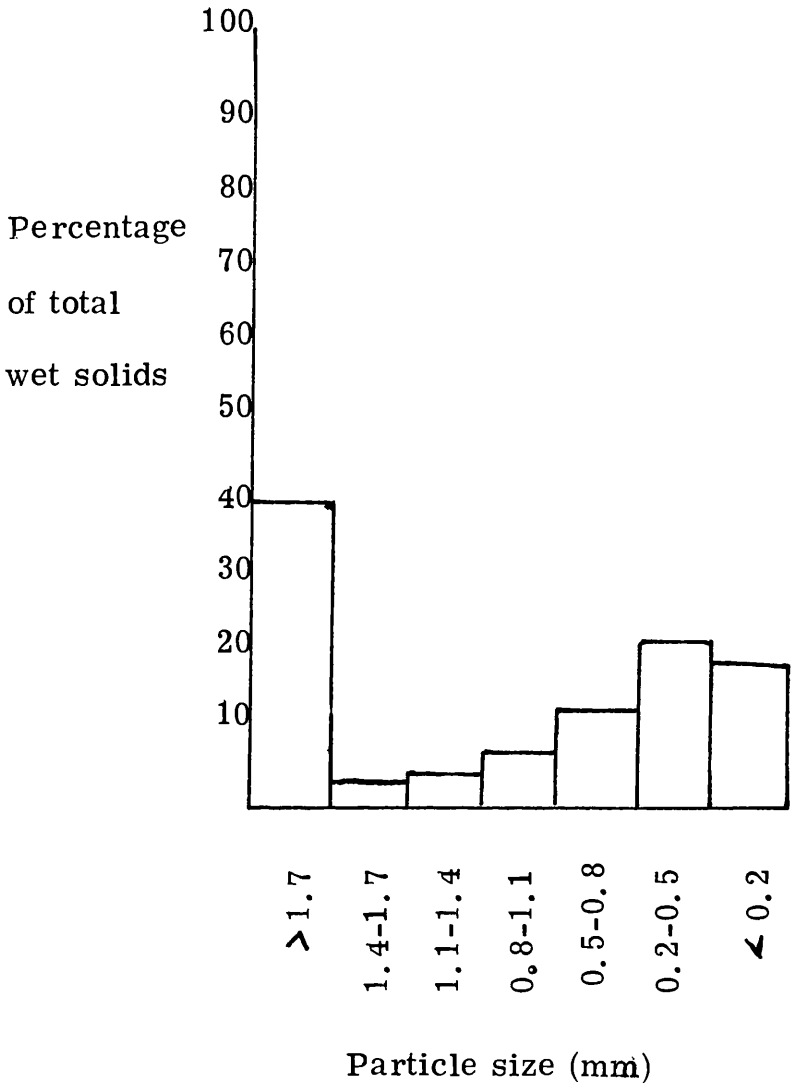



Figure 24. The distribution of particle size (wet) in ground kibbled carob used for growth experiments.

is 64.5%, confirming the findings of the gravimetric analysis viz. the kibbled carob has a high content of soluble solids.

Production of tannase kibbled carob slurries

Although a substantial reduction in the tannin content of carob residues fermented by M1 was observed, as mentioned earlier, no tannase could be detected in the supernatants of these flasks.

The effects of ammonium sulphate and carob concentrations on kibbled carob slurries with Aspergillus niger M1.

The effects of these two parameters on protein yields are shown in the histograms, figures 25-28, with their accompanying base plans. The total yield of protein per flask is shown in fig. 25. Yields in excess of 700 mg protein/flask, equivalent to 14 g TCPL, could be obtained. By subtracting the protein contents of the control flasks (which were uninoculated) from these yields, the de novo protein production, due to fungal synthesis was calculated. This is shown in figure 26. From figure 26, two main facts emerge: (1) when nitrogen is limiting, as is assumed when 100% of the added ammonium sulphate is utilised (see figure 29), the protein yields increased with increasing carob concentration to a maximum for the particular concentration of ammonium sulphate supplied. When concentrations of ammonium sulphate greater than 1% were used, a change in profile occurred. The protein yield increased with increasing

Figure 25. Yields of total protein produced (mg/flask) in kibbled carob slurries fermented by Aspergillus niger M1. Base plan below, with flasking giving highest yields ringed ; histogram right.

% carob

| % ammonium sulphate | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| 2.0 | 95 | 230 | 385 | 440 | 485 | 630 | 725 | 400 | 385 |
| 1.5 | 85 | 230 | 365 | 475 | 575 | 700 | 735 | 680 | 385 |
| 1.25 | 105 | 210 | 395 | 480 | 585 | 630 | 619 | 600 | 575 |
| 1.0 | 105 | 240 | 295 | 390 | 535 | 550 | 625 | 600 | 525 |
| 0.75 | 75 | 210 | 275 | 375 | 415 | 530 | 575 | 570 | 525 |
| 0.55 | 105 | 230 | 275 | 320 | 345 | 360 | 355 | 450 | 465 |
| 0.35 | 105 | 150 | 215 | 220 | 245 | 260 | 280 | 350 | 375 |

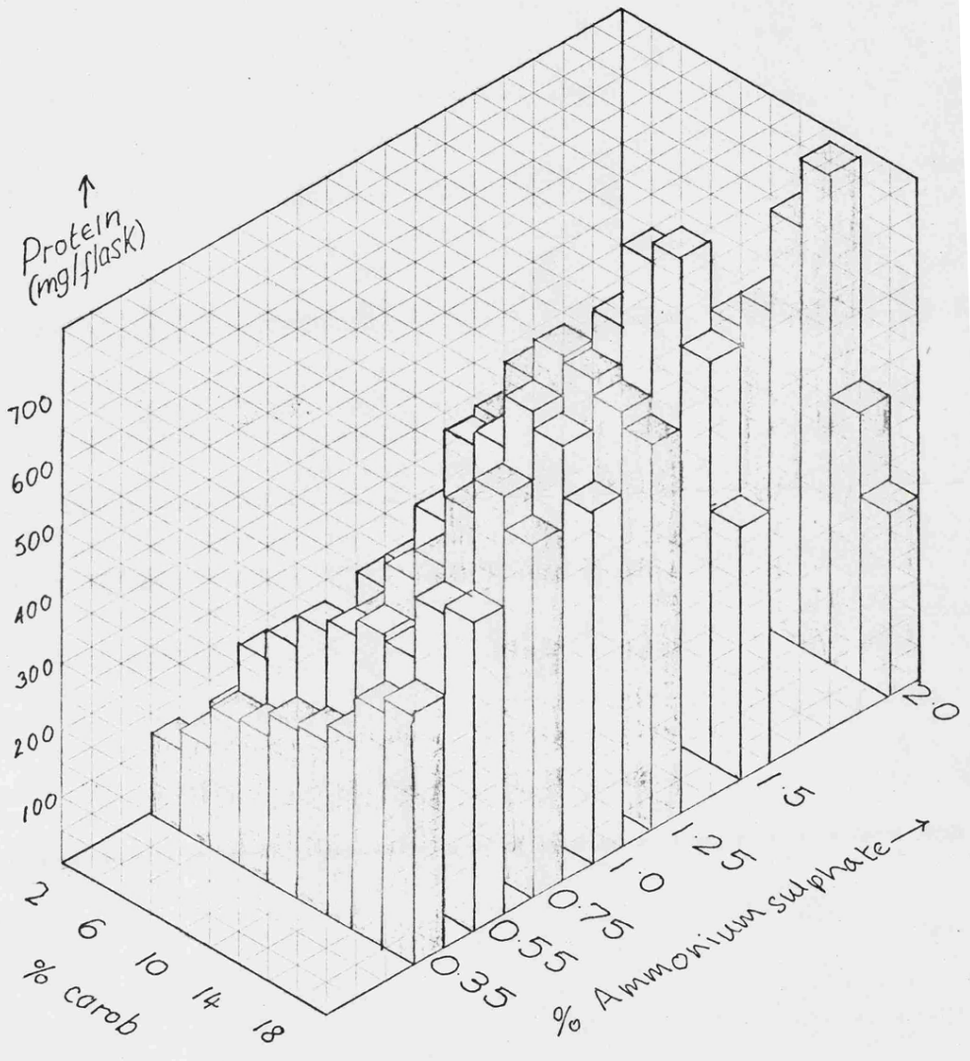
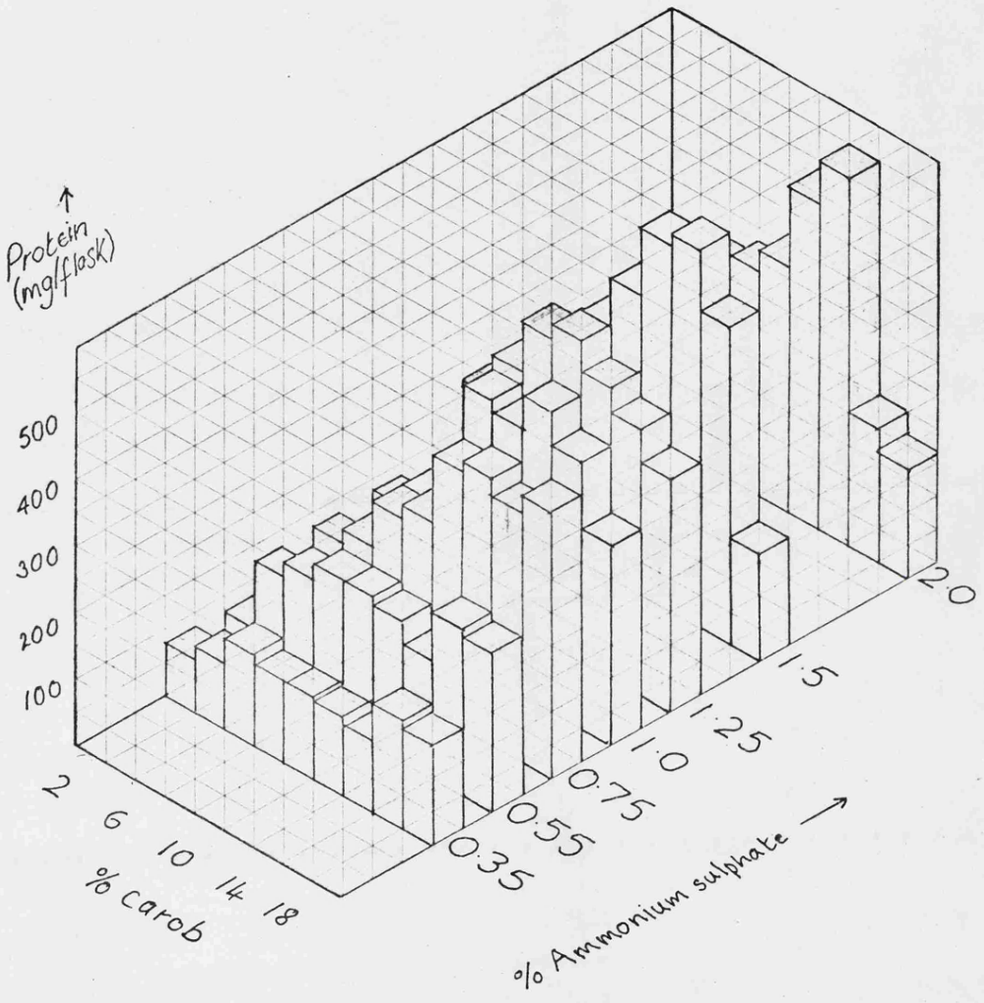

Protein (mg/flask)
700
600
500
400
300
200
100
2
6
10
14
18
% carob
0.35
0.55
0.75
1.0
1.25
1.5
2.0
% Ammonium sulphate

**Figure 26.** Protein yields (mg/flask) in kibbled carob slurries fermented by Aspergillus niger M1. Base plan below, with flasking giving highest yields ringed; histogram right.

| % ammonium sulphate | % carob | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 |
| 2.0 | 70 | 180 | 310 | 340 | 360 | 480 | 550 | 200 | 160 |
| 1.5 | 60 | 180 | 290 | 375 | 450 | 550 | 560 | 480 | 160 |
| 1.25 | 80 | 100 | 320 | 380 | 460 | 480 | 440 | 400 | 350 |
| 1.0 | 80 | 190 | 220 | 290 | 410 | 400 | 450 | 400 | 300 |
| 0.75 | 50 | 160 | 200 | 275 | 290 | 380 | 400 | 370 | 400 |
| 0.55 | 80 | 180 | 200 | 220 | 220 | 210 | 180 | 250 | 240 |
| 0.35 | 80 | 100 | 140 | 120 | 120 | 110 | 105 | 155 | 150 |


Protein (mg/flask)
500
400
300
200
100
2
6
10
14
18
% carob
0.35
0.55
0.75
1.0
1.25
1.5
2.0
% Ammonium sulphate

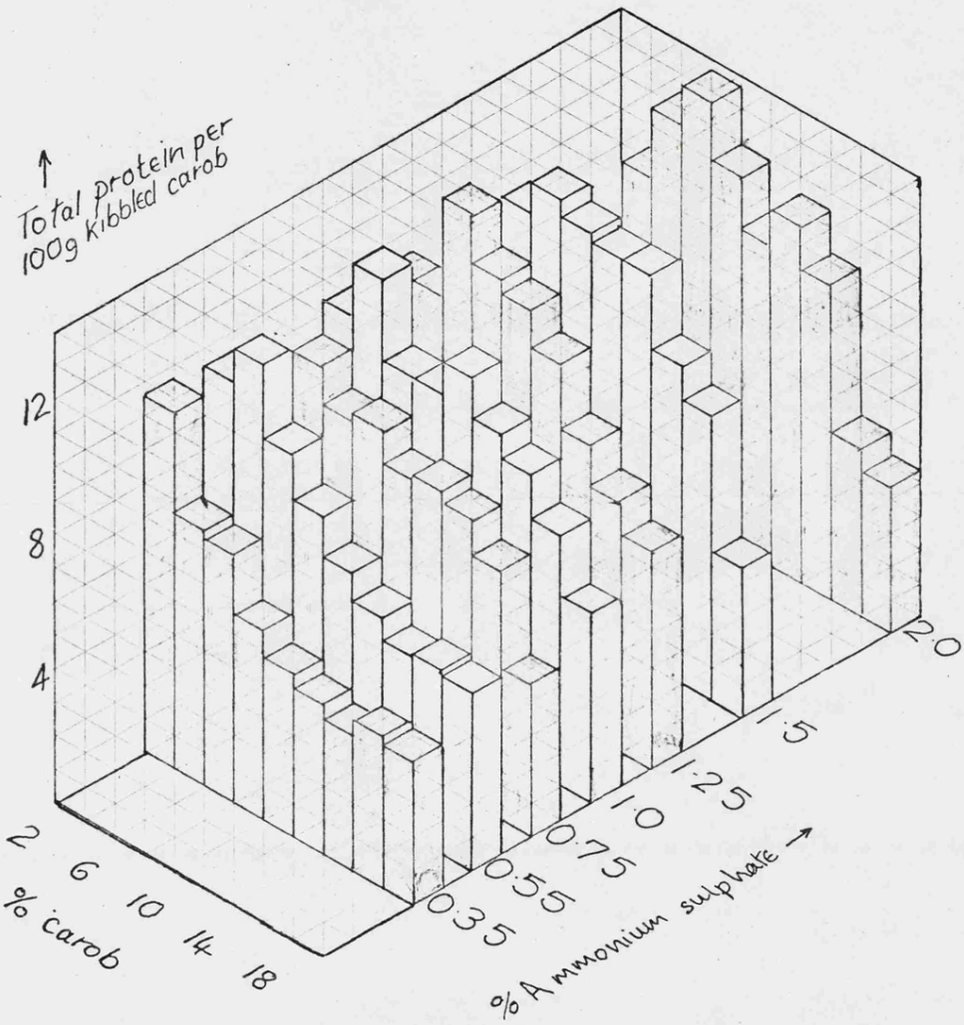
carob concentrations to a maximum but then decreased with further increases of carob concentration. This fall-off in protein yields with the higher carob and ammonium sulphate concentrations produced a sharp "dip" in the far right hand corner of figures 25 & 26. (2) The maximum synthesis of protein occurred in flasks with 10-12% carob and 1.5-2.0% ammonium sulphate.

The relationship between the efficiency of protein production (g total protein produced/100 g carob supplied) and the ammonium sulphate concentrations produced an entirely different shape to the histogram (figure 27). Here, maximum protein efficiencies were generally produced in low carob concentrations, with a reduction in efficiency as the carob concentration was increased. The maximum protein efficiencies for each ammonium sulphate concentration were all of the same order but with the highest efficiencies produced in flasks with 6-8% carob and 1.25-2% ammonium sulphate. As was observed with the previous figures 25 and 26, there was a pronounced dip in the shape of the figure when high carob and ammonium sulphate concentrations were employed.

The appearance of the histogram when the protein contents of fermented residues are plotted against the ammonium sulphate and carob concentration (figure 28) was very similar to the protein efficiency graph, figure 27. The maximum

Figure 27. Yields of total protein produced per 100g kibbled carob supplied, in kibbled carob slurries fermented by Aspergillus niger M1. Base plan below, with high yielding flasks ringed; histogram right.

| % ammonium sulphate | % carob 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| 2.0 | 9.5 | 11.5 | 12.8 | 11.0 | 9.7 | 10.5 | 9.1 | 5.0 | 4.3 |
| 1.5 | 8.5 | 11.5 | 12.2 | 11.9 | 11.5 | 11.6 | 9.2 | 8.5 | 4.3 |
| 1.25 | 10.5 | 10.5 | 13.2 | 12.0 | 11.7 | 10.5 | 8.7 | 7.3 | 6.4 |
| 1.0 | 10.5 | 12.0 | 9.8 | 9.7 | 10.7 | 9.2 | 8.9 | 7.3 | 5.8 |
| 0.75 | 7.5 | 10.5 | 9.2 | 9.4 | 8.3 | 8.8 | 8.2 | 7.1 | 4.7 |
| 0.55 | 10.5 | 11.5 | 9.2 | 8.0 | 6.9 | 6.0 | 5.1 | 5.6 | 5.2 |
| 0.35 | 10.5 | 7.5 | 7.2 | 5.5 | 4.9 | 4.3 | 4.0 | 4.4 | 4.1 |


Total protein per 100g kibbled carob
12
8
4
2 6 10 14 18
% carob
0.35 0.5 0.75 1.0 1.25 1.5 2.0
% Ammonium sulphate

protein contents of the residues occurred when 4-10% carob and 1.25-2.0% ammonium sulphate were supplied. The lower the carob/ammonium sulphate ratio for any ammonium sulphate concentration the greater was the protein content of the residue. Once again, in flasks where high carob and high ammonium sulphate concentrations were employed, there was a dip in the histogram.

The percentage utilisation of the added ammonium sulphate is shown in figure 29. In flasks with low ammonium sulphate concentrations, 100% utilisation of the ammonia could be achieved by using a suitably high carob concentration. However, when ammonium sulphate was supplied at levels in excess of 1.25% ammonium sulphate was not completely utilised in any of the carob concentrations tested. Indeed, there was a decrease in the percentage utilisation of ammonia with increasing ammonium sulphate concentrations between 1.25 and 2.0%.

The total sugars present in the supernatant, however, were efficiently used at all concentrations of ammonium sulphate tested (fig.30). There was a trend of a decrease in utilisation of sugars with an increase in carob concentration. Once again, in those flasks with high carob and ammonium sulphate concentrations, the low percentage utilisation was observed, giving a sharp dip in the shape of the histogram.

Figure 28. Percentage protein contents of fermented residues in kibbled carob slurries fermented by Aspergillus niger M1. Below, base plan with high yielding flasks ringed; histogram right.

% carob

| % ammonium sulphate | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| 2.0 | 22.3 | 26.1 | 28.8 | 24.2 | 21.4 | 24.0 | 22.7 | 12.4 | 10.8 |
| 1.5 | 19.8 | 26.2 | 25.6 | 25.0 | 25.1 | 20.1 | 22.9 | 23.7 | 10.4 |
| 1.25 | 24.8 | 25.0 | 28.6 | 26.0 | 26.5 | 22.1 | 20.3 | 19.7 | 14.2 |
| 1.0 | 24.2 | 20.3 | 24.8 | 22.4 | 21.5 | 19.3 | 19.4 | 18.4 | 13.7 |
| 0.75 | 19.3 | 21.3 | 20.1 | 23.0 | 19.3 | 17.3 | 18.7 | 19.2 | 16.1 |
| 0.55 | 25.0 | 16.2 | 20.1 | 19.2 | 16.7 | 15.7 | 13.0 | 14.4 | 13.9 |
| 0.35 | 20.6 | 25.0 | 15.7 | 12.5 | 11.7 | 11.5 | 11.4 | 11.5 | 11.4 |

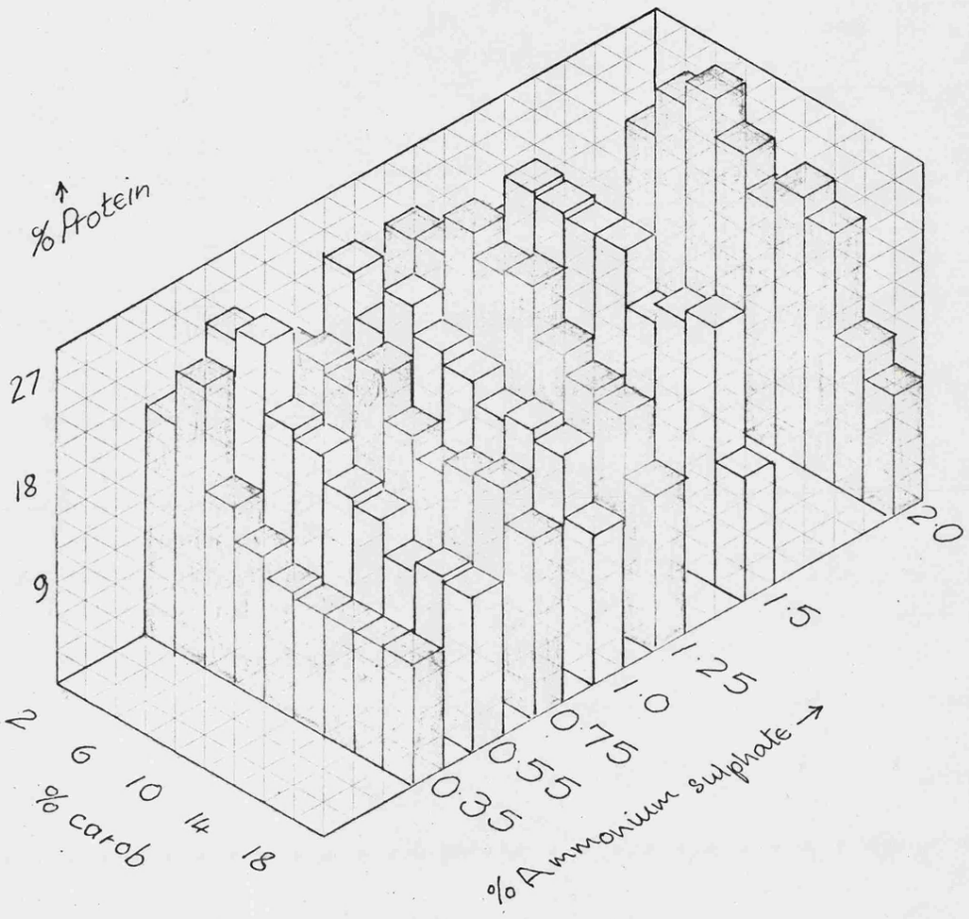

% Protein
27
18
9
2
6
10
14
18
% carob
0·35
0·5
0·75
1·0
1·25
1·5
2·0
% Ammonium sulphate

Figure 29. The percentage utilisation of ammonia in kibbled carob slurries fermented by Aspergillus niger M1. Base plan below, histogram right.

| % ammonium sulphate | % carob | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 |
| 0.35 | 50 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0.55 | 35 | 60 | 80 | 90 | 100 | 100 | 100 | 100 | 100 |
| 0.75 | 35 | 40 | 65 | 85 | 95 | 100 | 100 | 100 | 100 |
| 1.0 | 30 | 50 | 80 | 90 | 95 | 100 | 100 | 95 | 70 |
| 1.25 | 10 | 20 | 30 | 45 | 65 | 70 | 75 | 70 | 55 |
| 1.5 | 15 | 20 | 35 | 50 | 70 | 75 | 80 | 75 | 25 |
| 2.0 | 5 | 20 | 25 | 30 | 25 | 35 | 40 | 30 | 25 |

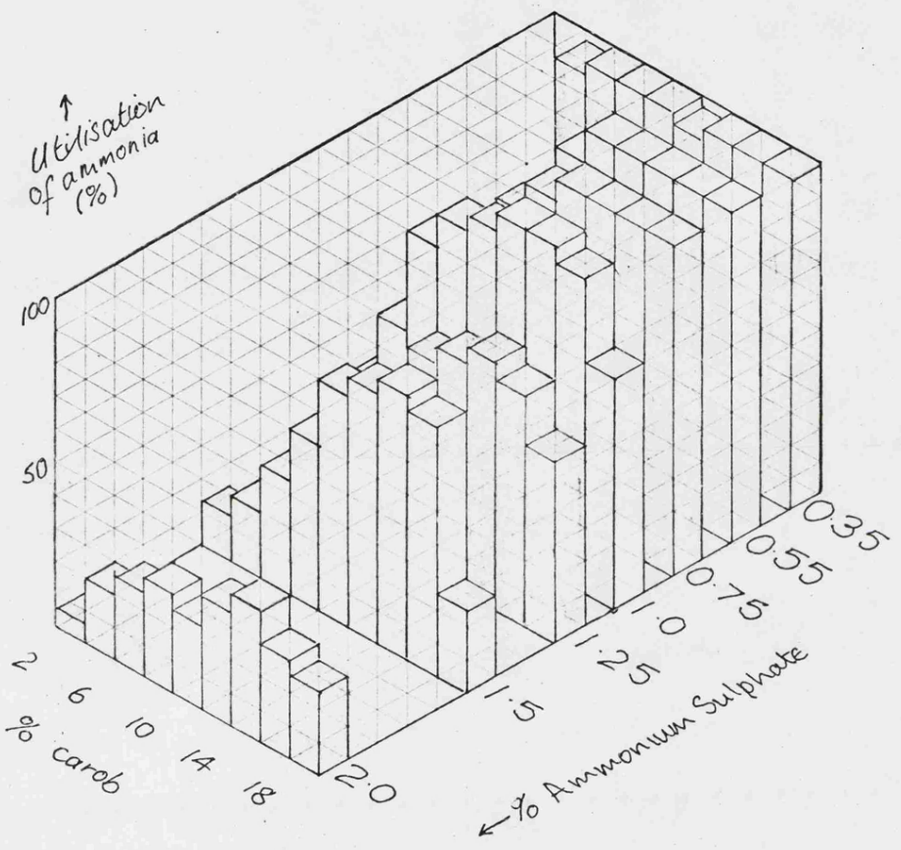
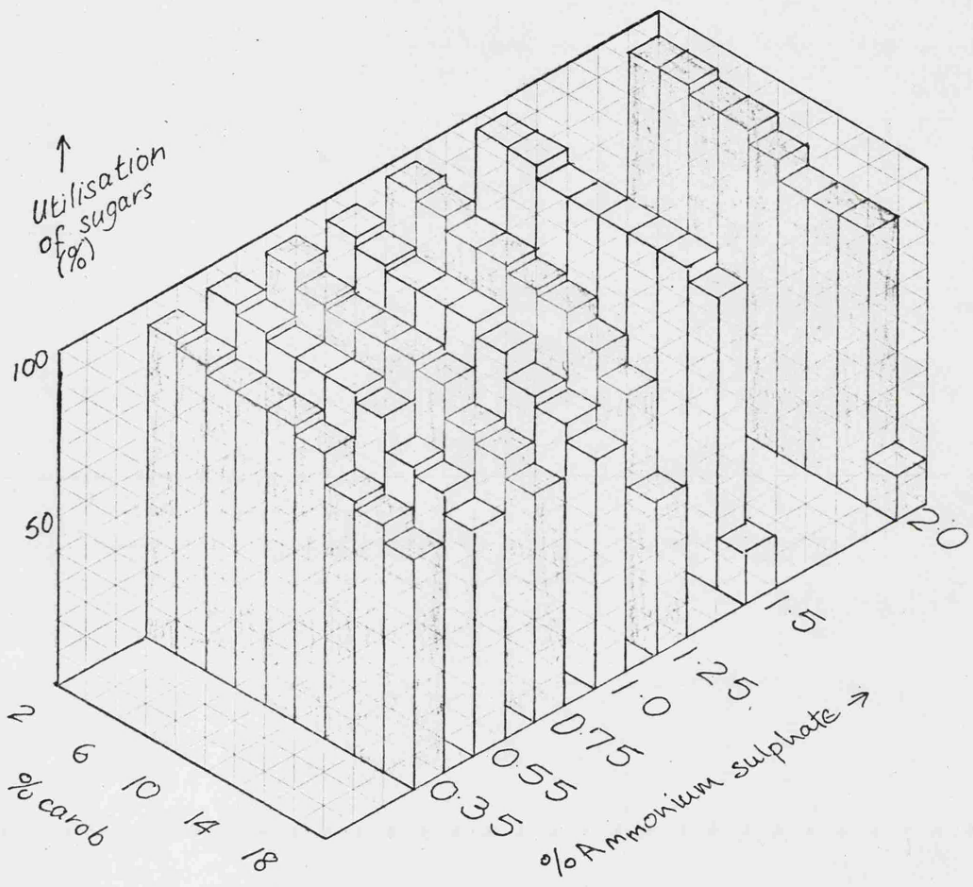

Utilisation of ammonia (%)
100
50
2
6
10
14
18
% carob
.5
1.0
1.5
2.0
0.35
0.55
0.75
% Ammonium Sulphate

Figure 30. The percentage utilisation of total sugars in kibbled carob slurries fermented by Aspergillus niger M1. Below, base plan. Histogram right.

| % ammonium sulphate \ % carob | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| 2.0 | 92 | 92 | 90 | 90 | 86 | 80 | 80 | 80 | 16 |
| 1.5 | 92 | 92 | 90 | 90 | 90 | 90 | 90 | 85 | 16 |
| 1.25 | 95 | 92 | 90 | 90 | 90 | 85 | 80 | 72 | 48 |
| 1.0 | 92 | 90 | 88 | 88 | 88 | 85 | 75 | 72 | 69 |
| 0.75 | 92 | 90 | 88 | 88 | 88 | 85 | 75 | 72 | 68 |
| 0.55 | 92 | 90 | 88 | 88 | 88 | 85 | 75 | 72 | 69 |
| 0.35 | 92 | 90 | 88 | 88 | 88 | 85 | 75 | 72 | 69 |


Utilisation of sugars (%)
100
50
2
6
10
14
18
% carob
0·35
0·55
0·75
1·0
1·25
1·5
2·0
% Ammonium sulphate

# DISCUSSION

Composition of carob pods

Since spent carob was thought to have a high cellulose content, the production of SCP from a carob based fermentation would best be achieved by using cellulolytic fungi. However, it was evident from the initial estimate of the cellulose content of spent carob afforded by the Updegraff method that it contained only 14% cellulose. Indeed, from the gravimetric analysis based on the Jermyn and Isherwood method, cellulose was not the largest carbohydrate component. The Updegraff method for cellulose determination depends on a vigorous acid digestion procedure to dissolve non-cellulosic carbohydrates which are then removed in the supernatant after centrifugation of the reaction mixture (Crampton and Maynard (1937)). The cellulose is then hydrolysed with sulphuric acid and estimated as sugar. This acetic acid/nitric acid digestion does not dissolve the cellulose, as shown by the fact that when a pure form of cellulose had been subjected to the acetic-nitric acid digestion reagent it had an identical sugar content to cellulose which had not undergone the digestion step.

A more extensive analysis of carob was afforded by applying the gravimetric method of Jermyn and Isherwood. The cellulose content of spent carob estimated by the gravimetric method agrees with that obtained by the

Updegraff method. A large part (37%) of the spent carob was solubilised by the combination of ethanol and water extractions. More than 50% of this solubilised material could be accounted for as sugars. The ethanol extract of spent carob contained 100% sugars, but the water extract had less than 50% attributable to sugars. Jermyn and Isherwood (1957) in their studies of pears ascribe the sugars extracted in the water extraction to "pectic substances". Other water soluble materials which would be extracted by the boiling water treatment are tannins and proteins - the former material would give rise to the brown colouration observed in the water extract.

The delignification procedure is considered as a particularly gentle procedure for removing lignin without loss of cellulose (Wise et al, 1946). This was also confirmed by experiments here. During the delignification procedure, at no time did the cellulose content of the holocellulose diminish, even after maximum solubilisation (as revealed by weight loss) of the lignin had occurred. It must be remembered however, that since the Jermyn and Isherwood analysis is a gravimetric analysis any non-lignin material remaining after ethanol and water extraction, but which is solubilised during the delignification procedure would be erroneously estimated as lignin, producing an over estimate of the lignin fraction. Indeed, the lignin contents of spent and kibbled carob were higher than expected

for the fruit of a legume which has no extensive visible lignification. The percentage of lignin in the carob pod would be increased after the water extraction process employed by Tate & Lyle for the extraction of the sugars in the pod, as was confirmed in the gravimetric analysis. The high content of lignin in spent carob would lower the nutritional value of the pod, since lignin is of little nutritional value itself (Crampton and Maynard, 1937).

Kibbled carob contained 5.5% cellulose when estimated by the Jermyn and Isherwood and Updegraff techniques. Charalambous (1966) has estimated the crude fibre content of kibbled carob to be 5.3%. Since the crude fibre is the loss in weight on ignition of material which is insoluble in 1% NaOH and 1% sulphuric acid (AOAC 1965, c) it is therefore only a very crude estimate of "cellulose", but in this case agrees remarkably well with the cellulose content. Results obtained in this study show that kibbled carob had a very high sugar content (61%), higher than that obtained by Charalambous (1966). Since the spent carob contained 23% sugars, the extraction process employed by Tate & Lyle only removes two thirds of the sugar present in kibbled carob.

Ball-milling the carob did not greatly increase the extractable sugars. Previous work with cellulosic materials (solka floc, Kat and Reese, 1968; wood,

Pew, 1957), showed that by subjecting the material to the action of a vibrator mill, an increase in soluble sugars could be attained. In agreement with this, the small increase in sugars observed on ball-milling the carob was matched with a corresponding decrease in cellulose and hemicellulose contents, both of which might be expected to produce sugars if partial depolymerisation occurred during the milling procedure. However, ball-milling did substantially increase the amount of material which was estimated as lignin. Increased solubility of the lignin fraction of sawdust after ball-milling has also been reported by Hulme and Thomas (1970).

When the cellulose contents of the material at each stage in the Jermyn and Isherwood analysis (back-calculated from the cellulose content of the whole material and the weight loss incurred at each stage) were compared with the cellulose content for each fraction obtained by the Updegraff method, a close agreement was generally obtained. However, an exception was found when the final product of the Jermyn and Isherwood analysis, $\alpha$-cellulose, was compared with the cellulose estimate by the Updegraff method. The latter method showed $\alpha$-cellulose contained only 70% "cellulose" - yet the actual (Updegraff) cellulose contents agreed with calculated cellulose contents, when based on the $\alpha$-cellulose content for the material from preceding stages in the fractionation procedure. One possible explanation of this paradox is that the -cellulose is partially solubilised

by the acetic acid-nitric acid delignification procedure employed in the Updegraff technique, such that 30% of it is dissolved and therefore only 70% estimated as cellulose. This could, for instance, arise if the ∝-cellulose had a low degree of crystallinity. As only the∝ -cellulose had a low "Updegraff cellulose" content, the affect of subjecting Whatman cellulose powder to the procedure for preparing ∝ -cellulose from holocellulose-KOH extraction - was investigated in the hope that this too might be rendered partially soluble to the Updegraff procedure. However, this hypothesis was unsubstantiated since the cellulose obtained after alkali treatment was not more soluble in the acid digestion procedure employed in the Updegraff method than untreated cellulose.

Tannin contents of carob pods

The two methods for estimating tannin gave comparable results. It was found that the one extraction of material with boiling water as employed by the AOAC was insufficient to extract the tannin exhaustively. Three extractions were deemed necessary. Interestingly, material which had been autoclaved had a higher apparent tannin content than unautoclaved material. The condensed tannins (polymers of catechins or similar flavins, Lewis and Starkey, 1969), in which ripe carob pods are particularly rich (Nachtomi and

Alumot, 1963) might need a more extensive extraction procedure than simpler "hydrolysed" tannins (gallotannic acid linked to glucose, Lewis and Starkey, 1969), which would account for the vast increase in apparent tannins when carob is autoclaved. Borstein et al, (1963) found that autoclaving kibbled carob failed to diminish its growth-inhibitory effect when used as a feed, but when the carob was subjected to a hot water treatment a marked improvement in its nutritional value occurred. In the studies reported here, both the hot-water and autoclave treatments removed tannin from the carob, but autoclaving produced an increase in the apparent tannin content of the carob perhaps by depolymerising condensed tannins, and therefore would be a harmful procedure if tannins were inhibitory to animal growth. It can be concluded that the two-stage hot water extraction of kibbled carob as used by Tate & Lyle to remove the sugars, and so producing spent carob,does not remove all the tannin. The tannin incidentally, is still present in levels sufficient to inhibit the germination of many fungal spores (Cook and Tabenhaus, 1911) which might be used for SCP production.

Selection of organism

The three main fractions present in spent carob suitable for fungal growth are : sugars, cellulose and lignin. Of the

three, lignin is by far the most recalcitrant; as fungal utilisation is both slow and incomplete (Cooke, 1957), perhaps indicating its unsuitability as a substrate for SCP production. However, as spent carob does contain large amounts of lignin, efforts were made to select organisms able to bring about its degradation by enrichment cultures of carob/soil mixtures and subsequent isolation on selective media containing a variety of phenolic compounds, inhibitory to most fungi except basidiomycetes. These attempts were unsuccessful, mainly due to the lack of inhibitory effect shown by the "selective" media towards certain fast growing fungi, which promptly swamped the agar plates. Production of SCP based on the utilisation of the other fractions, sugars and cellulose, would seem a more viable commercial proposition. For a process based on the utilisation of sugars in the carob, Aspergillus niger M1 was an obvious choice, since it had already been shown to produce high yields of protein on carob sugars and was undergoing extensive toxicity tests by Tate & Lyle. Another useful attribute of this organism is that it is able to reduce the tannin content of spent carob during its fermentation, as reported in this thesis. For the fermentation of spent carob it would be an advantage if the organism was able to consume

the cellulose fraction as well as the sugars. Accordingly, the "cellulolytic cultures" supplied by Tate & Lyle were screened for the ability to grow on carob agar. It should be emphasised that these fungi were deemed cellulolytic on the basis of their ability to solubilise phosphoric acid-swollen cellulose, and not native cellulose - and so their potential cellulolytic activity should be viewed with caution (Rautella and Cowling, 1966 and Wood, 1969). The fungi that grew well on this carob agar were screened for cellulolytic activity. The organism chosen for further studies was M223, Trichoderma koningii. Aspergillus niger does not produce a complete cellulase system, although it does possess enzymes and can degrade hemicelluloses (Tsujasaka et al, 1971, Clark and Stone, 1965).

Enzyme studies with Aspergillus niger and Trichoderma koningii.

The reason why Trichoderma koningii M223 failed to grow in submerged culture in shake flasks containing 1% tannic acid or 2% spent carob, but would grow on carob as a solid substrate fermentation, is not immediately apparent. Trichoderma strains have often been cultured in shake flasks (e.g. in bran based media: Neuderoffer and Smith, 1970); so inadequate aeration of the slurry cultures is unlikely to be the cause. Perhaps significantly, the fungus did show a very small amount of growth at the air-liquid interface, around the perimeter of

flasks containing 2% spent carob, suggesting that aeration is a key factor. It might be therefore that these observations are peculiar to media containing tannin. Aspergillus niger M1 produced a tannase with a pH optimum similar to that of the strain used by Yamada et al (1968).

In spite of a reduction in the tannin content of spent carob during its fermentation by Aspergillus niger M1, no tannase could be detected in the culture filtrate. Possible explanations are that the enzyme was produced intracellularly, although it produces the enzyme extracellularly in semi-synthetic media with tannin as the only carbon source, or that the assay system was swamped by the presence of large amounts of tannin released during autoclaving of the carob. Indeed, the assay mixture was rendered very opaque, at the wave length tested, by the addition of culture filtrate, suggestive of the presence of large amounts of tannin.

It is still not clear whether fungi need to produce phenol oxidases to degrade lignin, although these enzymes are frequently found in fungi actively degrading lignin (Kirk, 1971). However, neither of these organisms produced detectable catecholase activity.

As already mentioned, Aspergillus niger has not been found to produce true cellulase activity, that is, to produce

glucose from native forms of cellulose. On the other hand, Trichoderma koningii rapidly utilised cellulose for growth and its enzyme system has been extensively investigated (Wood, 1968; Hallwell and Riaz, 1970). The strain investigated here, M223, produced cellulase when grown on cotton wool as a substrate. M223 cellulase had an optimum pH of 4.8, in common with the cellulases of many other fungi (Wood, 1970) including the others tested here. Production of cellulase was inhibited initially in cultures containing glucose, until the glucose had been consumed. This agrees with other evidence for catabolite repression of cellulase by glucose (Hulme and Stranks, 1970; Nisiwaza et al, 1972.) It might therefore be assumed that cellulase would not be produced by M223 in a fermentation of spent carob until the carob sugars are utilised. However, in a fermentation system such as a solid substrate fermentation, sugar exhaustion may occur locally in the vicinity of the fungus, whilst material not attacked by the fungus has an unaltered sugar content; thus when an extract of the whole substrate is prepared it could contain both cellulase (from sugar depleted areas) and sugar (from zones furthest from the fungal activity). However, cellulase

activity from solid substrate fermentation was detected only when the sugar level had fallen to a constant value when very small quantities of cellulase were detected. The lack of extensive cellulose degradation might be due to the high tannin content of spent carob exerting an inhibitory effect on cellulase action, as found by Benoit and Starkey in their studies on the effect of tannin on cellulose decomposition in the soil (Benoit and Starkey, 1967; Starkey and Benoit, 1967). During the course of screening for cellulase extracts from solid substrates fermentation on which M223 had been growing, an enzyme was detected which could produce glucose from the culture filtrate concentrate under the conditions of the cellulase assay. The substrate for this enzyme must also therefore be present in this ammonium sulphate precipitated concentrate. This production of glucose was not through the action of a tannase (on tannic acid), since treating the concentrates with gelatin, which would precipitate tannin, did not decrease the activity of the enzyme-substrate mixture.

It was interesting to observe that Trichoderma koningii M223 when grown in cotton wool media, produced much more cellulase in flasks without glucose than in those to which glucose had been added. During the metabolism of this glucose, M223 produced a marked decrease in the pH, reducing it to a value below that of the optimum for

cellulase activity, pH 4.8. It could be therefore that cellulase activity, although produced earlier in cultures with glucose than without, is inhibited from reaching high levels by the low pH produced through the metabolism of the sugar.

Fermentations

There are numerous parameters to consider when assessing the commercial value of an SCP process, the most important probably being cost. From the literature survey it is also apparent that there are numerous parameters on which to base fungal yields in an SCP process. The whole purpose of an SCP process is to produce protein, and so yields expressed in terms of protein are of more value than those expressed solely as mycelial yields. From an economic point of view there may be little reason for comparing protein yields calculated per gram of sugar consumed, since this gives no information as to whether any sugar remains in the culture broth after fermentation. A further misrepresentation is achieved if the yields are based on reducing sugars consumed when other sugars are present, for the fungus may well have used these other sugars in addition to, or in preference to, the reducing sugars. Thus it can be argued that figures based on total sugar supplied will

have more weight when comparing SCP processes.
On the other hand, if sugars are only one of the utilisable fractions of the substrate, (e.g. as in carob), then protein yields based on sugars alone would give misleadingly high values. In this case it might be better to base the figures on total carbohydrate or, as carbohydrates may not be the only material consumed for protein synthesis, on weight of substrate supplied. Another parameter supplying a somewhat different expression ofyields, is the TCPL (Total Crude Protein per Litre). This gives an indication of the efficiency of the culture system; for a system giving 10g TCPL would obviously yield more protein in one harvest operation (which might be expensive) than a system yielding only 5g TCPL. With all these parameters it is essential to indicate the time taken to produce the yields since generally, the longer the fermentation period, the more power is consumed, and the greater is the expense incurred.

Also one must be extremely wary in comparing protein yields of systems in which different methods of protein estimation have been made. As mentioned earlier, the most common method has been by multiplying the Kjeldhal nitrogen by 6.25. Each method has its own particular advantages and disadvantages, and can give widely differing results (Gorsuch

and Norton, 1969). Methods based on summation of amino acids produced on hydrolysis of the protein are subject to least error, but for routine purposes where large numbers of samples have been assayed, the Kjeldhal method used in this work was found satisfactory. The obvious drawback of the Kjeldhal nitrogen x 6.25 method is that non-proteinaceous nitrogen is estimated as protein. One of the largest components of non-proteinaceous nitrogen in mycelial fungi is due to chitin. Solomons (1973) found that a fungus (not named) used for SCP production contained 11.8% N which is equivalent to 70% protein (Kjeldhal nitrogen x 6.25). However, further analyses revealed that this nitrogen comprised only 75% $\alpha$-amino nitrogen, the remainder being chitin (10%) and nucleic acid (15%). On summation of amino acids the "true" protein content was found to be 50%. Other estimates (Spensley et al, 1972) have put the non-proteinaceous nitrogen as high as 30% of the total nitrogen. The chitin content of Aspergillus niger cell walls has been found to vary from 8 to 21% when grown over an eight day period (Blumenthal and Roseman, 1957). By using a sodium hydroxide extraction step, as used in the work reported here, a large source of error was removed from the estimation of protein by Kjeldhal x 6.25,

since chitin was not solubilised during the extraction process. It was possible to estimate the chitin content of A. niger M1 based upon the difference in nitrogen content of mycelium with and without sodium hydroxide extraction which, if assumed to be due to chitin alone, was 11.8%, within the range found by Blumenthal and Roseman (1957) for their strain of A. niger. The second drawback of using Kjeldhal N x 6.25 is that the factor 6.25 is based on the assumption that the fungal protein contains 16% nitrogen (as for many cereal grains and feeding stuffs, Litchfield, 1968). For instance, one estimate on mushroom mycelium from the fruiting body of Agaricus campestrus (Fitzpatrick et al, 1946) puts the nitrogen content of the protein as low as 11.8%.

Finally, when comparing the industrial value of different SCP processes, not only the protein yields per gram of substrate should be taken into account, but also the dilution of the substrate necessary for the fermentation and the BOD of the process effluent. Most of the substrates reported in the literature have been used in concentrations of 4-8%, necessitating large volumes of fresh water which can be both expensive to acquire and, after the fermentation, to dispose of (Litchfield, 1968). In this environment

conscious age, necessary treatment of an SCP effluent to lower the BOD could be a costly process, and so a system that produces a fermentation liquor of low BOD might have distinct economic advantages.

Bearing these factors in mind an attempt can be made to compare the different carob fermentations reported here with (a) each other, and (b) other SCP processes reported in the literature.

The utilisation of spent carob for SCP production

The fermentation of spent carob by M1 produced a residue with a low protein content. When compared with other slurry fermentations (Tables 10 and 11) comparable yields of protein per 100 g substrate supplied and TCPL were obtained but the percentage protein of the residue was very low, too low in fact, for the requirement for a pig feed for which this SCP is intended. (A pig feed should contain a minimum of 16-20% protein ARC, 1967). It was significant to observe that the yield of protein obtained from spent carob slurries using M1, could be increased if glucose was added to stationary-phase cultures, indicating that M1 is unable to utilise the remaining fraction of the carob.

Efforts to render the carob more susceptible to fungal attack failed. Aspergillus niger did not produce more protein

* yields based on reducing sugar, but non-reducing sugar also present.

protein calculated by Lowry method.

Table 10. Protein yields from liquid fermentations

| Substrate | Organism | g/Protein/ 100g substrate /sugar supplied | TCPL g/L | % Protein | Days Incubation | Authors |
|---|---|---|---|---|---|---|
| 1. Molasses | Basidiomycetes | 1-21 | 0.2-9 | 20-54 | 8 | Reusser et al. 1958 |
| Molasses | Rhizopus sp. | 5.4 | 8 | 25 | 7 | Shukla and Dutta 1960 |
| 2. Sulphite liquor | Basidiomycetes | 2-16 | 0.3-6 | 15-38 | 10 | Reusser et al 1958 |
| 3. Cheese whey | Morchella spp. | 8 | 4-6 | 35 | 5 | Litchfield and Overbeck, 1965 |
| 4. Vinasse | Basidiomycetes | 34* | 4-6 | 23-28 | 12 | Falanghe 1962 |
| 5. Soya bean whey | Basidiomycetes | 34-49* | 2-3 | 34-54 | 3-8 | Falanghe et al. 1964 |
| 6. Citrus press water orange juice | Agricus blazei | 10 | 5-6 | 32.5 | | Block et al. 1953 |
| 7. Canning wastes | Morchella spp. | 7-14 | 0.2-4 | 26-50 | | Litchfield and Overbeck 1965 |
| Carob Liquor | A. niger M1 | 9.4 | 6.6 | 25-35 | 2-3 | Tate & Lyle QR 1970 3, 120. |

Table 11. Protein estimated by Kheldhal x 6.25

except :

1. Protein determined by summation of amino acids
2. Protein determined by Biuret method
3. Protein determined by modified Kjeldhal method.

Table 11. Protein yields from slurry fermentations

| Substrate | Organism | g protein per 100g substrate | TCPL g/L | % Protein in residue | % Days Incubation | Authors |
|---|---|---|---|---|---|---|
| 1. Sweet potato | Cladosporium sp | 31.2 | 3-4 | 39 | 4 | Gray & Abou El Seoud 1966 |
| 2. Cassava | Cladosporium sp | - | 2 | 20 | 4 | Gray & Abou El Seoud 1966 |
| 3. Whole beets | Cladosporium sp | 3.2-3.9 | 2-3 | 29 | 4 | Gray & Abou El Seoud 1966 |
| 4. Beet shreds | Cladosporium sp | 7.6 | 3.8 | 10 | 4 | Gray & Abou El Seoud 1966 |
| 5. Rice | Trichoderma sp | 21 | 4.6 | 30 | 4 | Gray & Karie 1967 |
| 6. Woodpulp | Rhizoctonia sp | 7.6 | 1.6 | 16-18 | 5 | Chahal and Gray 1969 |
| 7. Cellulose | Aspergillus fumigatus | 6.5-10[1] | [1] | 4-13[1] | 4 | Rogers et al 1972 |
| 8. Cellulose | Myrothecium verucaria | 3.5[2] | 1.42 | 5/10[2] | 6 | Updegraff 1972 |
| 9. Barley | Rhizopus sp Aspergillus sp | 14-17 | 5.0 | 47 | 1½ | Reade et al 1972 |
| Carob (spent) | A. niger M1 | 10[3] | 2.0[3] | 15[3] | 3 | Ibid |
| Carob (kibbled) | A. niger M1 | 11[3] | 13.5[3] | 27[3] | 3 | Ibid |

when grown on spent carob which had been ball-milled for 18 hours, than it did on coarsely ground carob. Ball-milling the carob might have been expected to increase the yield of protein produced during its fermentation by M1 on the grounds that (a) the carob particle size is smaller and therefore a larger surface area is presented to the fungus for a more extensive enzymic degradation, (b) certain fractions, e.g. cellulose are depolymerised by this treatment and that by milling the lignin fraction is often dissociated from the ligno-cellulose complex making the cellulose fractions more susceptible to enzymic attack (Pew and Weyna, 1962). Pre-treating the carob with dilute alkali failed to increase the yields of M1, yet other workers found such a treatment with cellulosics beneficial to the yield of fungus (Rogers et al, 1972) and digestibility by fungal enzymes in vitro (Moore et al, 1972). It was found that M1 was able to utilise alkali treated carob (if only after prolonged incubation) when it had been washed free of alkali, but not when the alkali had been neutralised with acetic acid. Other work using acid hydrolysed sisal wastes which were subsequently neutralised, also failed to support the growth of M1 or Trichoderma koningii (Tate & Lyle, 1972,c). Fifty per cent of the sugar content of the spent carob was lost during the alkali and washing treatments which,

unless recovered, constitute another disadvantage to the alkali treatment of carob in preparation for fermentation. The high sugar content of spent carob, when contrasted with substrates which have been treated successfully with alkali (straw, cellulose and wood, Takrow and Feist, 1969, Rogers et al, 1972, Moore et al, 1972) is thus a major factor in evaluating the suitability of carob for an alkali-swelling treatment.

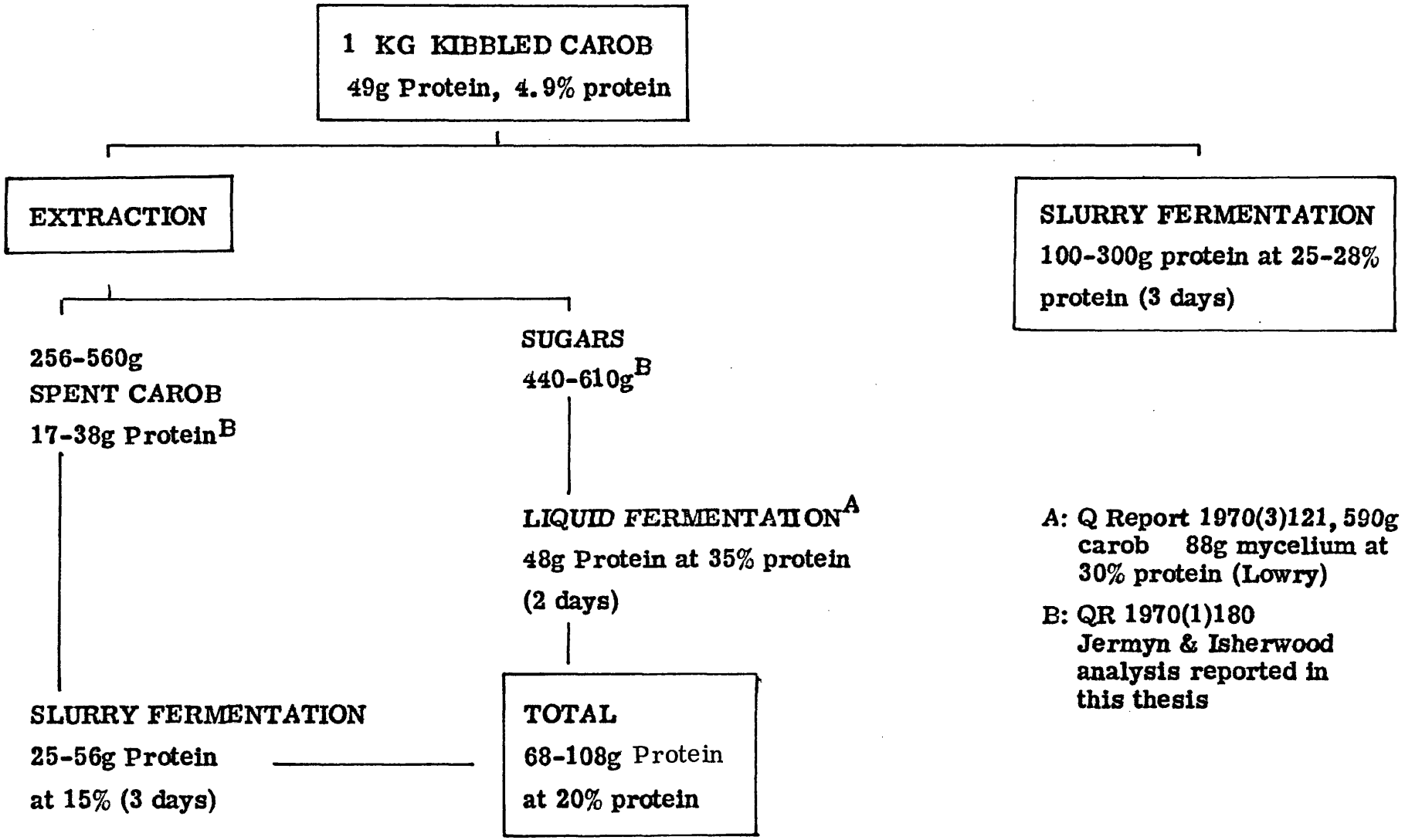
Disappointingly, M223 Trichoderma koningii, which had been shown to produce large amounts of cellulose when grown on cotton wool, failed to produce significant amounts of cellulase in solid substrate fermentations of spent carob. In spite of a substantial reduction in sugars and ammonia during the growth of M223 there was no significant increase in the protein content. It seems likely therefore that since growth was plainly visible on the solid substrate, the carob assuming a green hue (due to the copious production of spores), the fungal protein produced was diluted by residual substrate.

The one-step fermentation of kibbled carob : a better proposition.

From the gravimetric analysis, it was revealed that kibbled carob contained a large water soluble fraction - including 60% sugars. Thus it was conceived that a single step slurry fermentation of kibbled carob would produce high

yields of protein with little dilution of the fermented material by residual, unfermented carob. Subsequent experimentation substantiated this belief.

A more efficient conversion of kibbled carob into protein can be achieved in a single slurry fermentation than when a two stage process is used (figure 31). Yields of protein could be obtained in concentrations of 700 mg/flask, equivalent to 14 g TCPL, well in excess of other fermentations reported in the literature, as shown in Tables 10 and 11. However, in these cited SCP systems, initial substrate concentrations were much lower than were used for kibbled carob slurries. Indeed, in the SCP fermentations shown in Tables 10 and 11, no effort was made to procure maximum protein yields by increasing the substrate concentration. The efficiency of the kibbled carob fermentation was such that 12-13 g of protein could be produced for every 100 g of carob supplied. Once again, this compares favourably with other slurry fermentation systems, although many liquid fermentations yielded greater efficiencies (Tables 10 and 11). The protein content of a proposed SCP feed must be above the minimum required for the animal to make use of it, e.g. 16% protein for pig feeds (ARC, 1967). From the data reported here, residues containing 20% protein could be easily achieved,




A: Q Report 1970(3)121, 590g carob 88g mycelium at 30% protein (Lowry)

B: QR 1970(1)180 Jermyn & Isherwood analysis reported in this thesis

Figure 31. Flow chart of possible fermentation routes for

and most of the flasks yielded residues with protein content in excess of 16%. The maximum protein content of the residues, 29%, obtained in those slurry fermentations with M1 is higher than most of those obtained in other slurry SCP systems, but lower than many of the liquid fermentations which, of course, are solely the protein content of the harvested mycelium. Pure Aspergillus niger mycelium was, in fact, found to contain 35% protein (Kjeldhal N x 6.25). It must be remembered that in liquid fermentations systems there is no residual solid substrate to dilute the fungal protein produced during fermentation.

Quantity or quality?

Which statistic would be better for an SCP process, a high TCPL or a high substrate conversion efficiency? If the cost of harvesting was of prime consideration then it might be advantageous to harvest a system producing maximal TCPL's. In this case, 12-14% carob, with 1.25-1.5% ammonium sulphate would be suitable. If, on the other hand, maximum conversion efficiencies were sought, then 6-8% carob with 1.25-1.5% ammonium sulphate would be more suitable. However, the latter conditions produced only 30-50% utilisation of ammonium sulphate. Greater utilisation of the ammonium sulphate (75%) can be achieved, coupled with a substrate conversion efficiency of 78% of the maximum,

when conditions optimal for maximum TCPL production are employed, i.e. 12.14% carob and 1.25-1.5% ammonium sulphate. Sugar consumption remained approximately at 85-90% for both sets of conditions.

Disadvantages of thick slurry fermentations

More power might be needed to aerate a 12-14% carob slurry, than is needed for 2% spent carob slurries or a liquid fermentation, especially since the kibbled carob slurry fermentation started with a solid density of 40 g /L ending at 70 g/L whilst the spent carob slurry and liquid fermentations ended with densities of 20 g/L and 40 G/L respectively.

It was apparent that when high kibbled carob and ammonium sulphate concentrations were employed a depression in growth occurred. Flasks containing these high concentrations were observed to assume a gel-like consistency at the end of the fermentation, making the shaking action of the orbital-shaker ineffective in distributing the contents of the flasks up the sides of the vessels. Therefore, it is highly likely that these flasks would suffer from inadequate aeration - a factor which could be responsible for the observed

depression in growth. Gray and Abou El Seoud (1966c) also observed a difficulty in aerating slurry cultures with a high solid content with their beet-shred fermentations.

From a physiological point of view, increasing the carob concentration results in an unavoidable increase in slurry density which makes it difficult to extract information on the carob-nitrogen effects, since any effect due to an increase in the carob concentration would be inseparable from a density effect. However, it would be possible to design experiments to provide information on the carbon-nitrogen effect by fixing the carob concentration and altering the nitrogen concentration.

Interestingly, Johnson (1952) found that poor aeration in the culture of fungi for penicillin produced high economic coefficients (EC). The EC is the weight of mycelium produced 1 g of sugar consumed. EC's are usually quoted when the sugar is the only carbohydrate supplied to the fungus, when the index is a measure of the capacity of the fungus to convert sugar into cell substance. In the case of kibbled carob slurries, the sugar measured in slurry supernatants may not be the only carbon source available to the fungus, and so EC's calculated on a sugar

basis might give misleading high values. However, it is interesting to observe that the situation Johnson observed also applies to the kibbled carob slurries, in that EC's of 0.5-0.7 were obtained in conditions assumed of poor aeration (16-18% carob, 15-20% ammonium sulphate) and EC's of 0.4-0.5 were obtained in flasks which gave the highest TCPLs. As already discussed, maximum protein efficiencies were not produced under conditions of poor aeration.

The future of the carob project

As for the future of the carob project, a number of points which emerge from these studies seem pertinent. (1) A solid substrate fermentation system in its simplest form, as used in these studies, is unsuitable for the production of fungal protein due to dilution of the protein synthesised by unfermented substrate. Similar conclusions were drawn by Young (1973) in his studies on fermentation of cassava. However, before discarding solid substrates fermentations completely, it would be advisable to examine a mixing and aeration process, e.g. by a rotating drum with veins, for fermenting the substrate, in the light of its success for the production of fungal metabolites (Underkoffler, 1949, Hesseltine, 1972).

(2) Cellulolytic fungi, other than Trichoderma koningii M223, which failed to grow in spent carob slurries, might be screened for their ability to convert carob into protein. However, it would seem a more profitable venture, in the light of the high lignin content of the spent carob, to examine the growth of fungi which are both cellulolytic and lignolytic on spent carob.

(3) From the success of the kibbled carob slurry fermentations reported here, it would be reasonable to suggest that a larger scale fermentation of the single step process should be tried, obviating the need for a secondary fermentation on the spent carob. Feeding trials on A. niger M1 would not be wasted since the same organism is used for the kibbled carob slurry fermentation.

ACKNOWLEDGEMENTS

I must thank firstly the Science Research Council for providing the grant enabling me to work on the project; secondly, Tate & Lyle Ltd. for providing the raw material and an industrial atmosphere; and thirdly, Mr. R.C. Codner for invaluable guidance and constructive criticism. Finally, I must thank Miss J. Hobley for patiently typing from an illegible manuscript.

## REFERENCES


AMBERG, H.R. and CORMACK, S.F. (1957). Aerobic fermentation studies of spent sulphite liquor. Sewage Ind. Wastes, 29, 570.

ANDERSON, R.F. and JACKSON, R.W. (1958). Essential amino acids in microbial proteins. Appl. Microbiol. 6, 369.

AOAC (1965 a). Official methods of analysis of the association of official agricultural chemists. Tannin 14.048, p.219.

AOAC (1965 b). Official methods of analysis of the association of official agricultural chemists. Tannin 9.061, p.139.

AOAC (1965 c). Official methods of analysis of the association of official agricultural chemists. Crude fiber 22.038, p.332.

ARC (1967). The nutrient requirements of farm livestock. No. 3 Figs p.85. Agricultural Research Council, 1967.

ATKIN, C., WITTER, L.D. and ORDAL, Z.J. (1967). Continuous propagation of Trichosporon cutaneum in cheese whey. Appl. Microbiol. 15, 1339.

BARNES, T.A., EGGINS, H.O.W. and SMITH, E.L. (1972). Preliminary stages in the development of a process for the microbial upgrading of waste paper. Int. Biodet. Bull. 8, 112.

BATTACHARJEE, J.K. (1970). Micro-organisms as a potential source of food. Adv. Appl. Microbiol. 13, 139.

BENOIT, R.E. and STARKEY, R.C. (1967). Enzyme inactivation as a factor in the inhibition of decomposition of organic material by tannin. Soil. Sci. 105, 203.

BHUTIA, I.S., SHARMA, H.K. and BAJA, J.K. (1972). Inhibitory effects of polyphenols on endopolygalacturonase from Aspergillus niger. Zentralbl. Bakteriol. Parasitenk. Infection. Skr. Hyg. Abz. 127, 437.

BLOCK, S.S. (1965). Garbage composting for mushroom production. Appl. Microbiol. 13, 5.

BLOCK, S.S., STEARNS, T.W., STEPHENS, R.L. and McCANDLESS, R.F.J. (1953). Mushroom mycelium. Experiments with submerged culture. J. Agr. Food Chem. 1, 890.

BLOCK, S.S., TSAO, G. and HAN, L. (1965). Production of mushrooms from sawdust. J. Agr. Food. Chem 6, 923.

BLUMENTAL, H.J. and ROSEMAN, S. (1957). Quantitative estimation of chitin in fungi. J. Bacteriol. 74, 222.

BONDI, A. and MEYER, H. (1944). Digestibility studies with poultry. The digestibility of dura, carobs and inde fleshings. J. Agr. Sci. 34, 118.

BORNSTEIN, S., ALUMOT, E., MOKADI, S. NACHTOMI, E. and NAHARI, U. (1963). Trials for improving the nutritional value of carobs with chicks. J. Agr. Res. 13, 25.

BRADSTREET, R.B. (1965). Kjeldhal method for organic nitrogen. Academic Press, New York, London.

BRESSANI, R. (1968). In "Single Cell Protein" ed. R.I. Mateles and S.R. Tannenbaum p.90, M.I.T. Press, Cambridge, Mass.

BROOK, E.J., STANTON, W.R. and WALLBRIDGE, A.W. (1969). Fermentation methods for protein enrichment of cassava. Biotechnol. Bioeng. 11, 1271.

CALLIHAN, C.D. (1970). How engineers are putting microbes to work. Chem. Eng. 77, 160.

CHAHAL, D.S. and GRAY, W.D. (1969). Growth of cellulolytic fungi on wood pulp. I. Screening of cellulolytic fungi for their growth as wood pulp. Indian Phytopath. 22, 79.

CHAHAL, D.S., MUNSHI, G.D. and CHEEMA, S.P.S. (1969). Fungal protein production by Rhizoctonia solani when grown on cellulose as the sole source of organic carbon. P.N.A.S. (India) 39B, 287.

CHAHAL, D.S., SIDHU, B.S. and CHORA, A.K. (1972). Essential amino acid content of P. crustosum grown on rice flour as a carbon source. Lab. Dev. Part B. 10, 41.

CHARALAMBOUS, J. (1966). The composition and use of carob bean. Cyprus Agriculture and Research Institute, April, 1966.

CHURCH, B.D., NASH, H.A. and BROSZ, W. (1972). Use of fungi imperfecti in treating food processing wastes. Dev. Ind. Microbiol. 13, 30.

CIEGLER, A. and ULLEHOJ, E.B. (1968). Mycotoxins. Adv. Appl. Microbiol. 10, 155.

CIRILLO, V.P., CRESTWOOD, W.A., HARDWICK, O. and SEELEY, R.D. (1960). Fermentation process for producing edible mushroom mycelium. U.S. Patent 2,928,210.

CLARKE, A.E. and STONE, B.A. (1965). Properties of a 1-4 glucan hydrolase from Aspergillus niger. Biochem. J. 96, 802.

COLNER, R.C. (1969). "Methods in Microbiology" vol. 1, p. 427, Ed. J.R. Norris and D.W. Ribbons. Academic Press, London and New York, 1969.

COOK, M.T. and TAUBENHAUS, J.T. (1911). The relation of parasitic fungi to the contents of the cells of the host plants. 1. Toxicity of tannin. Delaware Agr. Sta. Bull. 91, 77 pp.

COOKE, W.B. (1957). Natural and induced fungal degradation of lignin. TAPPI 40, 301.

CRAMPTON, E.W. and MAYNARD, L.A. (1938). Cellulose and lignin in relation to animal feeds. J. Nutr. 15 (4), 385.

CRAWFORD, D.L., McCOY, E., HARKIN, J.M. and JONES, P. (1973). Production of microbial protein from waste cellulose by Thermonospora fusca, a thermophilic actinomycete. Biotech. Bioeng. XV, 833.

CRUZ, R.A., MALAISA, R.E., CRUZ, T.J. and PUSAG, C.C. (1967). Biological treatment of bagasse enhances its value as a soil conditioner or feed. Sugar News 43, 15.

DAVIES, W.N.L., ORPHANOS, P.I. and PAPACONSTANTINOU, J. (1971). Chemical composition of developing carob pods. J. Sci. Fd. Agric, 22, 83.

DEAN, E.W. and STARK, D.D. (1920). A convenient method for the determination of water in petroleum and other organic emulsions. J. Ind. Eng. Chem. 12, 486.

DEMAIN, A.L. (1971). Microbial production of food additives. Symp. Soc. Gen. Microbiol. 21, 77.

DOCTOR, V.M. and KERUR, L. (1968). Penicillium mycelium waste as protein supplement in animals. Appl. Microbiol. 16, 1723.

DONEFFER, E., ADELEYE, I. and JONES, T. (1969). Effect of urea supplementation on nutritive value of NaOH treated oat straw. Adv. Chem. Series 95, 328.

DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A. and SMITH, F. (1956). Colourmetric method for the determination of sugars and related substances. Anal. Chem. 28, 350.

DUNLAP, C.E. and CALLIHAN, C.D. (1969). Microbial protein production from sugar cane bagasse. Sugar J. July, p.13.

DUNLAP, C.E., HAN, Y.W. and CALLIHAN, C.D. (1970). Pilot plant fermentation of waste cellulosics in batch and continuous flow operation. Amer. chem. Soc. Div. Water, Air, Waste. Chem. Gen. Pap. 1970, 10, 191.

DUNN, C.G. (1952). Food yeast. Wallerstein Labs. Communs. 15, 61.

EL-NAWAWY, A.S. (1970). Utilisation of bagasse pith for the production of fodder yeast. Agr. Res. Rev. 48, 93.

EWESON, E.W. (1936). Yeast flow wood. Chem. Inds. 38, 573.

FALANGHE, H. (1962). Production of mushroom mycelium as a protein and fat source in submerged culture in a medium of vinasse. Appl. Microbiol. 10, 572.

FALANGHE, H., SMITH, K. and RACKIS, J.J. (1964). Production of fungal mycelium protein in submerged culture of soya bean whey. Appl. Microbiol. 12, 330.

FITZPATRICK, W.H., ESSELEN, W.B. and WEIR, E. (1946). Composition and nutritive value of mushroom protein. J. Am. Dietetic Assoc. 22, 318.

FOSTER, J.W. (1949 a). Nutritional value of fungus protein in "Chemical Activities of Fungi", p.106, Academic Press, New York, 1949.

FOSTER, J.W. (1949 b). Chemical nature of the mould mycelium in "Chemical Activities of Fungi", Academic Press, New York.

FOSTER, J.W. (1949 c). Spore composition in "Chemical Activities of Fungi", p. 85, Academic Press, New York.

FRASER, A.R. and RUSSEL, J.D. (1969). A spectrophotometric method for the determination of cation-exchange capacity of clay minterals. Clay Minerals 8, 229.

FREEMAN, R.R. (1964). Separation of cells from fluids. Biotechnol. Bioeng. 6, 87.

GADEN, E.L. (1964). Process and equipment design for less developed areas. In "Global impacts of applied microbiology", M.P. Starr ed. pp. 338-343. Wiley, New York.

GHOSE, T.K. (1969). Continuous enzymatic saccharification of cellulose with culture filtrates of Trichoderma viride. Biotech. Bioeng. XI, 239.

GHOSE, T.K. and KOSTICK, J.A. (1970). A model for continuous enzymatic saccharification of cellulose with simultaneous removal of glucose syrup. Biotech. Bioeng. XII, 921.

GORCICA, H.J., PETERSON, W.H. and STEENBOCK, H. (1935). Nutritional value of fungi III. Growth of rats on unsupplemented and supplemented mould proteins. J. Nutrition 9, 701.

GORSUCH, T.T. and NORTON, R.L. (1969). Determination of protein in biological materials and foodstuffs. J. Fd. Technol. 4, 1.

GRAHAM, V.E., GIESON, D.L. and LAWTON, W.C. (1953). Increasing the food value of whey by yeast fermentations. III. Pilot plant studies. Can. J. Technol. 31, 109.

GRAY, W.D. (1962). Microbial protein for the space age. Devel. Ind. Microbiol. 3, 63.

GRAY, W.D. (1966 a). Fungal protein for food and feeds. I. Introduction. Econ. Bot. 20, 89.

GRAY, W.D. (1966 b). Fungi and world protein supply in World Protein Resources. Adv. in Chem. series 57, 261.

GRAY, W.D. (1970). The use of fungi as food and in food processing. Critical Rev. Food Technol.

GRAY, W.D. and ABOU-EL-SEOUD, M. (1966 a). Fungal protein for food and feeds. II. Whole sweet potato as a substrate. Econ. Bot. 20, 119.

GRAY, W.D. and ABOU-EL-SEOUD, M. (1966 b). Fungal protein for food and foods. III. Manoic as a potential crude raw material for tropical areas. Econ. Bot. 20, 251.

GRAY, W.D. and ABOU-EL-SEOUD, M. (1966 c). Fungal protein for food and feeds. IV. Whole sugar beets or beet pulp as a substrate. Econ. Bot. 20, 372.

GRAY, W.D. and KARVE, M.D. (1966). Fungal protein for food and feed. V. Rice as a source of carbohydrate for the production of fungal protein. Econ. Bot. 21, 110.

GRAY, W.D., OCH, F.F. and ABOU-EL-SEOUD, M. (1964) Fungi imperfecti as a potential source of edible protein. Devel. Ind. Microbiol. 5, 384.

GRAY, W.D. and PAUGH, R. (1967). Fungal protein for food and feeds. VI. Direct use of cane juice. Econ. Bot. 21, 273.

GREENE, H.C. and FRED, E.B. (1934). Maintenance of vigorous mould stocks. Ind. Engng. Chem., ind. Edn. 20, 1297.

HALLIWELL, G. and RIAZ, M. (1970). The formation of short fibres from native cellulose by components of Trichoderma koningii cellulase. Biochem. J. 116, 35.

HAN, Y.W., DUNLAP, C.E. and CALLIHAN, C.D. (1971). Single cell protein from cellulosic wastes. Food Technol. 25, 32.

HARRIS, E.E., SEAMAN, J.F., MARQUAROT, R.R., HUNNAN, M.L. and ROGERS, S.C. (1948). Fodder yeast from wood hydrolysates and still residues. Ind. Eng. Chem. 40, 1220.

HARTLEY, R.D. and KING, N.J. (1973). Modified wood waste and straw components of animal feeds. J. Sc. Food Agr. 24, 495.

HEDENSKOG, G. and Van HOFSTEN, A. (1970). The ultrastructure of Spirulina platonsis, a new source of protein. Physiol. Plant, 23, 209.

HENIS, Y., TAGARI, H. and VOLCANI, R. (1964). Effect of water extracts of carob pods, tannic acid and their derivatives on the morphology and growth of micro-organisms. Appl. Microbiol. 12, 204.

HESSELTINE, C.W. (1965). A millenium of fungi, food and fermentation. Mycologia, 57, 149.

HESSELTINE, C.W. (1972). Solid state fermentations. Biotech. Bioeng. 14, 517.

HOOGERHEIDE, J.C. (1954) in "Industrial Fermentations" Vol. II, Eds. L.A. Underkoffler and R.J. Hickey, p.122, Chem. Publ. Co. New York.

HUANG, T.L., HAN, Y.W. and CALLIHAN, C.D. (1971). Application of the Lowry method for determination of cell concentration in fermentation of waste cellulosics. J. Ferm. Technol. 49, 574.

HULME, M.A. and STRANKS, D.W. (1970). Induction and regulation of production of cellulase by fungi. Nature, 226, 469.

HULME, M.A. and THOMAS, J.F. (1970). Erosion of wood by enzymes of wood rotting basidiomycetes. Bi-Mon. Res. Notes, Dept. Fish. Forest, Can. 20 (6), 57.

HUMFIELD, H. and SUGIHARA, T.F. (1949). Mushroom mycelium production by submerged propagation. Food Technol. 3, 355.

HUMFIELD, H. and SUGIHARA, T.F. (1952). The nutrient requirements of Agaricus campestris grown in submerged culture. Myocologia 44, 605.

HUMPHREY, A.E. (1970). Microbial protein from petroleum. Proc. Biochem. 5 (6), 19.

HUNT, R.S. and COBB, F.W. (1971). Selective medium for the isolation of wood-rotting basidiomycetes. Can. J. Bot. 49, 2064.

IIBUCHI, S., MINODA, Y. and YAMADA, K. (1967). Studies on tannin acyl hydrolase of micro-organisms II. A new method for determining the enzyme activity using the change of ultra-violet absorption. Agr. Biol. Chem. 31, 513.

INTERNATIONAL UNION OF BIOCHEMISTRY (1965). Enzyme nomenclature. p.39. Elseviar Publishing Co., Amsterdam.

JANARDHANAN, K.K., KAUL, T.N. and HUSAIN, A. (1970). Use of vegetable wastes for the production of fungal protein from Morchella species. J. Food Sci. Technol. 7, 197.

JARL, K. (1971). Utilisation of waste materials by fermentation with special reference to Symba Yeast Process. Socker. Handl. 25, 4.

JEFFERYS, G.A. (1948). Mould enzymes produced by the continuous tray method. Food. Ind. 20, 688.

JERMYN, M.A. and ISHERWOOD, F.A. (1956). Changes in the cell wall of the pear during ripening. Biochem. J. 64, 123.

JOHNSON, M.J. (1952). Recent advances in penicillin fermentation. World Health Organization, Monograph Ser. 10, 99.

KATZ, M. and REESE, E.T. (1968). Production of glucose by enzymatic hydrolases of cellulose. Appl. Microbiol. 16 (2), 419.

KIHLBERG, R. (1972). The microbe as a source of food. Ann. Rev. Microbiol. 20, 427.

KIRK, T.K. (1971). Effects of micro-organisms on lignin. Ann. Rev. Phytopath. 6, 185.

KIRK, T.K. and KELMAN, A. (1965). Lignin degradation as related to the phenoloxidases of selected wood destroying basidiomycetes. Phytopathology, 55, 739.

KLUG, M.J. and MARKOVETZ, A.J. (1971). The utilisation of aliphatic hydrocarbons by micro-organisms. Advan. Microbiol. Physiol. 5, 1.

KOSARIC, N. and LEDUY, A. and ZAJIC, J. (1973). Submerged culture growth of edible mushrooms on waste sulphite liquors. Can. J. Chem. Eng. 51, 186.

LEWIS, J.A. and STARKEY, R.L. (1969). Decomposition of plant tannins by some soil micro-organisms. Soil Sci. 107, 235.

LIPINSKY, E.S. and LITCHFIELD, J.H. (1970). Algae, Bacteria and yeasts as food or feed. Critical Rev. Food Technol. 1, 581.

LITCHFIELD, J.H. (1968). In "Single Cell Protein", ed. R.I. Mateles and S.R. Tannenbaum, p.309, M.I.T. Press, Cambridge, Mass.

LITCHFIELD, J.H., OVEREECK, R.C. and DAVIDSON, C. (1963). Factors affecting the growth of morel mushroom mycelium in submerged culture. J. Agr. Food Chem. 11, 158.

LITCHFIELD, J.H. and OVEREECK, R.C. (1965). Proceedings 1st International Congress on Food Science and Technology (London) 1962, vol. 12, pp. 511-520. Gordon and Broach Science Publishers, New York (1965).

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951). Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265.

MANDELS, M. and REESE, E.T. (1964). Fungal cellulases and the microbial decomposition of cellulosic fabric. Develop. Ind. Microbiol. 5, 5.

MANDELS, M., WEBER, J. and PARIZEK, R. (1971). Enhanced cellulase production by a mutant of Trichoderma viride. Appl. Microbiol. 21, 152.

MAYMONE, B. and BATTAGLINI, A. (1953). Nutritive value of exhausted carob bean pulp residual from the industrial preparation of alcohol. Ann. Sper. Agar. (Rome) 7, 1953.

MOLITORIS, H.P. (1963). Beauvaria tenella NRRL 2334, 2335, 2336, previously Ag. campestris. I. Systematics. Arch. Mikiobiol. 47, 57.

MOORE, W.E., EFFLAND, M.J. and MILLET, M.A. (1972). Hydrolysis of wood and cellulose with cellulolytic enzymes. J. Agr. Fd. Chem. 20, 1173.

MOUSTAFA, A.M. (1960). Nutrition and development of mushroom flavour in Agaricus campestris mycelium. Appl. Microbiol. 8, 63.

MULLER, W.R. (1949). Food yeast production in Germany. Milchwissenchaft, 4, 147.

NACHTOMI, E. and ALLMOT, E. (1963). Tannins and polyphenols in carob pods (Ceratonia siliqua). J. Sci. Food Agric. 14, 464.

NELSON, N. (1944). A photometric adaption of the somogyi method for the determination of glucose. J. Biol. Chem. 153, 375.

NEUDOERFFER, T.S. and SMITH, R.E. (1970). An evaluation of fungal enzymes for the solubilization of wheat bran constituents. Can. J. Microbiol. 16, 139.

NIELSEN, N., SANDEGREN, E. and LJUNGDAHL, L. (1949). Amino acids in Rhodotorula gracilis. Nature 164, 1055.

NISIZAWA, T., SUZUKI, H. and NISIZAWA, K. (1972). Catabolite repression of cellulase formation in Trichoderma viride. J. Biochem. 71, 999.

O'BRIEN, M.A. (1948) The control of humidity by saturated salt solutions. J. Sc. Instruments. 25, 73.

PATENT, (1969). Isolation of enzyme concentrates from microbiological fermentation studies by precipitation with tannin. G.B. 1 156 900 Arzeimittelwerk Dresden V.E.B. P 2.7.69, A 14.6.68 (28 422/68).

PATHAK, S.G. and SESHADRI (1965). Use of Penicillium chrysogenum as animal food. Appl. Micro. 13, 262.

PEPPLER, H.J. (1970). In "The Yeasts" Ed. Rose, A.H. and Hamson, J.J., Vol. 3, Yeast Technology, pp. 421-462. Academic Press, London and New York.

PERLMAN, D. (1965). The Chemical Enviroment for Fungal Growth. 2. Carbon Sources in "The Fungi I" Ainsworth G.C. and Sussman, A.S., Eds. Academic Press, 1965.

PEW, J.C. (1957). Properties of powdered wood and isolation of lignin by cellulolytic enzymes. T.A.P.P.I. 40, 553.

PEW, J.C. and WEYNA, P. (1962). Fine grinding 1. Enzyme digestion and the lignin cellulose band in wood. T.A.P.P.I. 45, 247.

PIDGEN, W.J. and HEANEY, D.P. (1969). Ligno cellulose in Ruminant nutrition. In Advance. Chem. Ser. 95, 245.

PLATT, B.S. (1964). Improvement of the nutritional value of foods and dietary regimens by biological agencies. Food Technol. 68.

POWELL, M.E. and ROBE, K. (1964). Yeast fermentation of whey cheese. Fd. process. 28, 80.

PRESCOTT, S.C. and DUNN, C.G. (1959). "Industrial Microbiology" 3rd Ed. McGraw-Hill Book Co. N.Y.(1959).

PRINGSHEIM, H. and LICHTENSTEIN, S. (1920). Versuche zur Anreicherung von Draftstroh mit Pilzeiweiss. Cellulosechemie 1, 29.

PROTEIN ADVISORY GROUP (1973). Proteins from micro algae and micro fungi. Tropical Science, 15, 77.

PURCHASE, F.H. and NEL, W. (1960). in "Biochemistry of some food borne microbial toxins". Eds. R.I. Mateles and G.N. Wogan, p.153 M.I.T. Press, Cambridge, Mass. and London.

RAO, N., NARAYANA, N., RAO, T.N. and RAMACHANDRA (1972). Nutritional evaluation of predigested protein rich food (miso like products). Effects of their supplementation to poor rice diet. J. Food. Sci. Technology. 9, 127.

RAUTELLA, G.S. and COWLING, E.B. (1966). A simple cultural test for relative cellulolytic activity of fungi. Appl. Microbiol. 14, 892.

READE, A.E., SMITH, R.H. and PALMER, R.M. (1972). The production of protein for non-ruminant feeding by growing filamentous fungi on barley. Biochem. J. 127, 32p.

REESE, E.T. (1963). Advances in enzymic hydrolysis of cellulose and related materials. American Chem. Soc. Pergamon. Press. p.1-33.

REGAN, R.W. and JERIS, J.S. (1970). A review of the decomposition of cellulose and refuse. Compost. Sci. 11, 17.

REUSSER, F., SPENCER, J.F.T. and SALLANS, H.R. (1958a). Protein and fat content of some mushroom grown in submerged culture. Appl. Microbiol. 6, 1.

REUSSER, F., SPENCER, J.F.T. and SALLANS, H.R. (1958b). Tricholoma nudum as a source of microbiological protein. Appl. Microbiol. 6, 5.

ROBINSON, R.F. and DAVIDSON, R.S. (1959). The large scale growth of higher fungi. Adv. Appl. Microbiol. 1, 261.

ROELAFSEN, P.A. and RHALENS, A. (1964). Changes in some B vitamins during moulding of soybeans by Rhizopus oryzeae in the production of tempeh kedelee. J. Food Sci. 29, 224.

ROGERS, C.J., COLEMAN, E., SPINO, D.F. and PURCELL, T.C. (1972). Production of fungal protein from cellulose and waste cellulosics. Enviro. Sc. Technol. 6, 715.

RUSSEL, P. (1956). A selective medium for the isolation of basidiomycetes. Nature 177, 1038.

SCRIMSHAW, N.S. (1968). Introduction in "Single cell protein". R.I. Mateles and S.R. Tannenbaum eds. pp.3-7 M.I.T. Press Camb. Mass.

SEARCY, R.L., REARDON, J.E. and FOREMAN, J.A. (1967). A new photometric method for serum urea nitrogen determination. Am. J. Med. Technol. 33, 15.

SHUKLA, J.P. and DUTTA, S.M. (1967). Production of fungal protein from waste molasses. Ind. J. Technol. 5, 27.

SMITH, R.H. and READE, A.E. (1973). Studies on a farm process for the production of fungal protein as a non-ruminant feed. J. Sc. Food Agric. 24, 496.

SNYDER, H.E. (1970). Microbial sources of protein. Adv. Food Res. 18, 85.

SOLOMONS, G.L. (1973). "Food from fungi". Microbiology group symposium. March, 1972. J. Sc. Food Agric. 24, 637.

SOMOGYI, M. (1937). A reagent for the copperiodometric determination of very small amounts of sugar. J. Biol. Chem. 117, 771.

SOMOGYI, M. (1952). Notes on sugar determination. J. Biol. Chem. 195, 19.

SPENSLEY, P.C., HALLIDAY, D. and ORR, E. (1972). Prospects for nonconventional protein resources. Trop. Sci. 14, 203.

SRINIVASAN, V.R. and HAN, Y.W. (1969). Utilisation of bagasse. Adv. Chem. Series 95, 447.

STANTON, W.R. and WALLBRIDGE, A. (1969). Fermented food processes. Proc. Biochem. (Apr) 45.

STARKEY, R.C. and BENOIT, R.F. (1968). Inhibition of decomposition of cellulase and some other carbohydrates by tannin. Soil Sci. 105, 291.

SWAIN, T. and HILLIS, W.E. (1959). The phenolic constituents of Prunus domestica . 1. The quantitative analysis of phenolic constituents. J. Sci. Food Agric. 10, 63.

SZUECS, J. (1954). Method of enhancing mushroom mycelium flavor. U.S. Patent 2,693,664 (1954).

SZUECS, J. (1956). Mushroom culture. U.S. Patent 2,761,246 (1956).

TAGARI, H., TAMIS, M. and VOLCANI, R. (1965). Effect of carob pod extract on cellulolyses, proteolysis, deamination, and protein biosysnthesis in an artificial rumen. Appl. Microbiol. 13, 437.

TAKATA, R. (1929). The utilisation of microorganisms for human food material. J. Soc. Chem. Ind. Japan 32, 243.

TAKROW, H. and FEIST, W.C. (1969). A method for improving the digestibility of ligno-cellulosic materials with dilute alkali and liquid ammonia. Adv. Chem. Series 95, 197.

TANNENBAUM, S.R. and MILLER, S.A. (1967). Effect of cell fragmentation on nutritive value of B. megaterium protein. Nature 214, 1261.

TATE & LYLE (1969a). Race plate experiments. Q.R. 1969, 1, 83.

TATE & LYLE (1969b). Biological evaluation of microbial protein. Q.R. 1969, 2, 121.

TATE & LYLE (1969c). Fermenter investigations. Q.R. 1969, 3, 106.

TATE & LYLE (1969d). Growth without supplementary nutrients. Q.R. (1969) 3, 111.

TATE & LYLE (1969e). Comparison of 50 L fermentor stage at 6% and 4% w/v Initial Total Sugar. Q.R. 1969, 3, 107.

TATE & LYLE (1969f). Effect of sugar concentration of a medium based on carob extract on the growth of A. niger Ml. Q.R. 1969, 3, 112.

TATE & LYLE (1969g). University of Athens, Microbial Protein Study. Q.R. 1969, 2, 104.

TATE & LYLE (1969h). Screening of microfungi. Q.R. 1969, 2, 112.

TATE & LYLE, (1969i). Cellulolytic microorganisms. Q.R. 1969, 2, 95.

TATE & LYLE (1969j). Solid substrate fermentation. Q.R. 1969, 3, 83.

TATE & LYLE (1969k). Semi solid and slurry fermentations. Q.R. 1969, 2, 119.

TATE & LYLE (1969l). Microbial decomposition of sugar exhausted carob meal. Q.R. 1969, 1, 82.

TATE & LYLE (1970a). Feeding trials. Q.R. 1970, 2, 151.

TATE & LYLE (1970b). Q.R. 1970, 3, 128.

TATE & LYLE (1970c). Fermenter investigations : "Scale up experiments". Q.R. 1970, 1, 182.

TATE & LYLE (1970d). Growth kinetic experiments with Penicillium sp. Q.R. 1970, 1, 187.

TATE & LYLE (1971). Product analysis. Q.R. 1971, 3, 3.

TATE & LYLE (1972a). Substrate screening (1) Orange waste. Q.R. 1972, 1, 15.

TATE & LYLE (1972b). Use of date waste as a slurry. Q.R. 1972, 1, 18.

TATE & LYLE (1972c). Substrate screening : sisal waste. Q.R. 1972, 1, 16.

THATCHER, F.S. 1954. Foods and feeds from fungi. Ann. Rev. Microbiol. 8, 447.

THAYSEN, A.C. 1956. Food and Fodder yeasts. In "Yeasts" W. Roman ed. pp. 155-210. Junk Publ. The Hague.

TSUJISAKA, Y., TAKENISH, S. and FUKUMOTO, J. (1971). Studies on hemicellulases. Part II. Mode of action of 3 hemicellulases produced from A. niger. J. Agric. Chem. Soc. Jap. 45, 253.

UNDERKOFLER, L.A., SEVERSON, G.M., GOERING, K.J. and CHRISTENSEN, L.M. (1947). Commercial production of mould bran. Cereal Chem. 24, 1-22.

UPDEGRAFF, D.M. (1969). Semimicro determination of cellulose in biological materials. Anal. Biochem. 32, 420.

UPDEGRAFF, D.M. (1970). Utilisation of cellulose from waste paper by Myrothecium verrucaria. Biotech. Bioeng. XIII, 77.

VAN BUREN, J.P. and ROBINSON, W.B. (1969). Formation of complexes between proteins and tannic acid. J. Agr. Food Chem. 17, 772.

VAN VEEN, A.G. and STEINKRAUS, K.H. (1970). Nutritive value and wholesomeness of fermented foods. J. Agr. Food Chem. 18, 576.

VELD HUIS, M.K. (1952). Reduction of organic matter in citrus-press liquor by aerated yeast propogation. Ind. Wastes. Practice Bull 57.

VINSON, L.J., CERE CEDO, L.R., MILL, R.P. and NORD, F.F. (1945). The nutritive value of Fusaria. Science, 101, 388.

WASSERMAN, A.E., HOPKINS, W.J. and PORGES, N. (1958). Whey utilisation. Growth conditions for Saccharomyces fragilis. Sewage and Ind. Wastes, 30, 913.

WILSON, R.E. (1921). Humidity control by means of sulphuric acid solutions, with critical compilation of vapour pressure data. J. Ind. and Eng. Chem. 13, 326.

WISE, L.E., MURPHY, M. and D'ADDIECO, A. (1946). Chlorite holocellulose, its fractionation and bearing on summative wood analysis and as studies on the hemicelluloses. Paper Trade J. 122, 35.

WOOD, T.M. (1968). Cellulolytic system of Trichoderma koningii. Biochem. J. 109, 217.

WOOD, T.M. (1969). The relationship between cellulolytic and pseudo cellulolytic micro-organisms. Biochem. Biophys. Acta, 192, 531.

WOOD, T.M. (1970). Cellulose and Cellulolysis. World Rev. Nutr. and Dietics. 12, 227.

WORGAN, J.T. (1960). The culture of higher fungi. Progress Ind. Microbiol. 8, 73.

YAMADA, H. , ADACHI, M., WATNABE, M. and SATO, N. (1968). Studies on fungal tannase I. Formation, purification and catalytic properties of tannase of Aspergillus flavus. Agr. Biol. Chem. 32, 1070.

YOUNG, B.V. (1972). Personal communication.